Evaluation of testicular oocytes and gonadal development in smallmouth bass (*Micropterus dolomieu*) under presumed and known exposure to estrogenic compounds

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ABSTRACT

Testicular oocytes (TOs), a form of gonadal intersex, have been found in black basses (*Micropterus* spp.) from many locations in North America. The presence of TOs is often assumed to imply exposure to estrogenic endocrine disrupting compounds (EDCs), however, a definitive causal relationship has yet to be established, and TO prevalence is not consistently low in fish from areas lacking evident EDC sources. This could suggest unknown or unidentified EDC sources, or that TOs can be stimulated by other stressors, or that they arise spontaneously during normal development. The goal of this dissertation research was to improve our ability to interpret observations of TOs in smallmouth bass (*M. dolomieu*) in particular, with approaches that are applicable to any other species. These approaches included statistical analysis of TO occurrence in wild populations where estrogenic exposure is more and less likely, and laboratory studies to characterize the early gonadal development in smallmouth bass, both in the presence and absence of exogenous estrogen. TO occurrence in smallmouth bass was assessed in eight watersheds in Northeastern Minnesota watersheds with differing degrees of human development, and hence, likelihood of EDC exposure. Testicular tissues from mature fish were evaluated using a semi-quantitative method that estimated TO density, normalized by cross-sectional area. TO prevalence and density among populations from more developed watersheds were higher than in populations from less developed watersheds. However, TO prevalence was unexpectedly high and variable (7-43%) in some populations from less developed watersheds, and only weak evidence was found for a relationship between TO density and watershed development, suggesting alternative or

more complex explanations for TO presence in smallmouth bass. To characterize early gonadal development in smallmouth bass, both in the presence and absence of estrogen, swim-up fry were exposed to control water or the potent model estrogen, 17α-ethinylestradiol (EE2), for 90 days, and reared for another 90 days in clean water. Development of gonadal tissue was assessed at 11 time points using standard histology methods. Ovarian differentiation was observed in 50% of control fish by test day 49, and testicular differentiation occurred by day 92. Among EE2-exposed groups, ovarian phenotypes were observed in 83% of fish by day 49, and in 98% of fish between days 92 and 180, indicating that EE2 can cause complete, and likely permanent, sex reversal in smallmouth bass. Among the EE2-exposed fish that developed ovaries between days 92 and 180, 38% had abnormal characteristics, suggesting that ovaries in sex reversed males can be distinguished from ovaries in genetic females. In a second laboratory study, smallmouth bass gonadal development was assessed during early life stage exposure across a range of concentrations of EE2, including environmentally realistic concentrations. 97% of fish exposed to 4.3 ng/L EE2 (mean measured concentration) had ovaries on test days 90 and 120, and 35% of ovaries in those groups had abnormal characteristics. Among control groups and fish exposed to lower concentrations (0.1 and 0.4 ng/L nominally, and 1.6 ng/L, mean measured concentration), only histologically normal ovaries and testes developed, in ratios that were not significantly different than 50:50. TOs were not observed in any control or EE2-exposed fish during these studies. This suggests that early life stage exposure to low concentrations of EE2 may not cause TOs in wild smallmouth bass, although the long-term outcomes of EE2 exposure in adult

bass are unknown. The results of these studies underscore the importance of understanding gonadal development in fish reared in both the absence and presence of exogenous estrogen for interpreting the occurrence of TOs in wild populations. Overall, the evidence produced by this research does not support the hypothesis that estrogenic compounds are the unique cause of TOs in wild bass. With current understanding, the occurrence of TOs in wild populations of smallmouth bass alone is not an appropriate biomarker for estrogenic exposure.

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I. Introduction

Estrogenic endocrine disrupting compounds (EDCs), including the endogenous hormones 17β-estradiol (E2) and estrone (E1), the synthetic estrogen 17α-ethinylestradiol (EE2), and hormone mimics like alkylphenols (e.g. octylphenol, nonylphenol) and bisphenol-A [1], are commonly detected in natural waters that receive inputs from wastewater treatment plants (WWTPs), industrial sites, and agricultural activities [2-4]. Estrogenic compounds have been associated with sexual disruption in many fish species in numerous laboratory studies [4]. Both juvenile and mature fish exposed to estrogenic compounds can develop biomarkers of endocrine disruption, including altered steroid hormone levels, elevated vitellogenin, feminized secondary sex characteristics, gonadal intersex, complete sex reversal, and decreased reproduction [5]. Intersex is considered abnormal in most non-hermaphroditic fish [6], and in males, may take the form of a few immature oocytes in otherwise normal testicular tissues (i.e. testicular oocytes, TOs), the presence of female gonadal structures (e.g. ovarian cavities, oviducts), or gonads with large, well developed regions of both ovarian and testicular tissue (i.e. mixed sex gonads). Sensitivity to gonadal disruption by exposure to estrogenic compounds varies by species. The lowest concentration of EE2 that has been reported to cause gonadal feminization in male fish of various species ranges from 0.2 ng/L (i.e. Chinese rare minnow, *Gobiocypris rarus* [7]) to 4 ng/L (i.e. fathead minnow, *Pimephales promelas* [8]; Table 1).

There is some evidence that TOs are a type of partial feminization that can be induced by exposure to concentrations lower than those that cause full sex reversal. For example, oocytes were observed in the testes of 22% and 64% of juvenile Chinese rare minnows exposed to 0.2 and 1 ng/L EE2, respectively, and 100% of fish exposed to 4 ng/L EE2 developed ovaries [7], indicating a pattern of increased severity of feminization with increasing concentration. Similarly, the percentage of male fathead minnows and rainbow trout (*Onchorhynchus mykiss*) with either intersex gonads or ovaries was positively correlated to early life EE2 exposure concentration [8, 9]. Some fish species may not respond to even high concentrations of EE2 with gonadal feminization, although this seems to be rare. For example, juvenile guppies (*Poecilia reticulata*) exposed to 10, 50, or 200 ng/L EE2 [10], and juvenile Western mosquitofish (*Gambusia affinis*) given 10 µg EE2 /g food did not develop testicular oocytes or sex reversal (Table 1; [11]).

Table 1. No-observed effect concentrations (NOEC) and lowest-observed effect concentrations (LOEC) for feminization of gonads in juvenile male fish exposed to 17α-ethinylestradiol.

Species	NOEC	LOEC	Reference
Chinese rare minnow *(Gobiocypris rarus)*	--	0.2 ng/L	Zha et al. 2008 [7]
Medaka *(Oryzias latipes)*	0.2 ng/L	2 ng/L	Balch et al. 2004 [12]
Zebrafish *(Danio rerio)*	1.67 ng/L	3 ng/L	Maack & Segner 2003 [13]
Fathead minnow *(Pimephales promelas)*	1 ng/L	4 ng/L	Lange et al. 2001 [8]
Roach (*Rutilus rutilus)*	0.3 ng/L	4 ng/L	Lange et al. 2008 [14]
Guppy (*Poecilia reticulate*)	200 ng/L	--	Nielsen et al. 2006 [10]
Western mosquitofish (*Gambusia affinis*)	10 μg/g[a]	--	Angus et al 2005 [11]

Endpoints were determined by histological observations in known or presumed males or by female-biased phenotypic sex ratios. [a] Administered via food

Gonadal development is highly sensitive to perturbation by exogenous hormones in early life stages, during the period of gonadal differentiation when germ cells and somatic cells are particularly responsive to hormonal signals. But even after sexual differentiation, the gonad may retain some degree of bipotentiality [6]. Therefore, exposure to estrogens outside of the most sensitive developmental period may result in either a moderate feminizing effect (e.g. TOs or mixed gonadal tissue), or a higher threshold for effects. For example, adult fathead minnows exposed to 100 ng/L EE2 for three weeks did not develop even moderately feminized gonads (i.e. intersex or TOs) [15]; whereas juvenile fathead minnows exposed to a much lower concentration of EE2 (4 ng/L) developed severely feminized gonads (i.e. sex reversal; [8]).

EE2 concentrations in natural waters appear unlikely to exceed 1 ng/L, even in low-flow conditions [16], although wild fish are potentially exposed to a complex mixture of estrogenic compounds. Estrogen receptor agonists are known to act in additivity [17], and to have similar effects on reproductive endpoints, including TOs [18]. The relative potency of EE2 is approximately 10 times greater than that of E2, which is 2-3 times more potent than E1, as measured by *in vivo* induction of vitellogenin (VTG; the female-specific egg yolk precursor protein) mRNA and *in vitro* estrogen receptor activation assays [19, 20]. BPA, octylphenol, and nonylphenol have estrogenic potencies that are one to several orders of magnitude lower than E2 [17, 20]. These wide ranges of estimated relative potencies are the result of the differing sensitivities of in vitro and in vivo assay systems. This implies that the level of total estrogenicity that fish are exposed to cannot be predicted with a high degree of confidence in our current state of

understanding. Furthermore, the additive and antagonistic effects of other classes of endocrine disrupting compounds that may be present in natural waters, like androgens, anti-androgens, and anti-estrogens, are also difficult to estimate.

Some wild fish populations appear to be more susceptible to gonadal anomalies than others. At least 54 wild species from 20 families worldwide have been reported with intersex [21]. It is interesting to note that several studies have found species with TOs coexisting in the same (presumably) contaminated habitat with species in which no TOs were found [22]. A survey of nine major river basins in the United States found that some male fish from four species had intersex gonads, out of 16 species evaluated, although sample numbers were small for some species [23]. The degree to which mild intersex is a part of the normal range of physiology is unknown for most species. The spontaneous occurrence of TOs in laboratory cultures of various model species has been estimated to range from <1% to 21% [24-27].

Despite these uncertainties, researchers have reported strong evidence that estrogenic compounds have caused reproductive disruption, including TOs, in wild populations of some species, notably white sucker (*Catostomus commersoni*), darters (*Etheostoma* spp.) and roach (*Rutilus rutilus*) exposed to wastewater treatment plant (WWTP) effluent [28-31]. Each of these case studies has documented a positive correlation between the degree and/or prevalence of intersex and the proximity to likely or confirmed sources of estrogenic compounds, as well as a consistently lower incidence of intersex in reference populations.

The black basses (Family: Centrarchidae, *Micropterus* spp.), have also been suggested as sentinel species for the presence of estrogenic compounds. Smallmouth bass populations with TO prevalence of up to 91% have been documented in several major U.S. river systems [23, 32-34]. This is often assumed to imply exposure to estrogenic compounds originating from human activities, because most of these observations were made in waters with known sources of estrogenic compounds, including WWTP outflows and agricultural activities. However, TO prevalence ranging from 7-60% has also been observed in bass populations in waters with no known sources of estrogenic compounds [33, 35], although true reference sites are not well represented among most of the *Micropterus* studies. Many of these studies either did not assess TO occurrence in bass near suspected EDC sources in comparison with reference sites [23, 32, 36], or reported that TO prevalence was not significantly lower at reference sites compared with sites near known EDC sources [33, 34, 37-40]. A single study reported weak evidence for an association between TOs and potential EDCs, citing a moderate correlation between TO prevalence and percent of agriculture in the watershed ($r^2 = 0.63$) [26]. However, correlations between TO prevalence and other watershed characteristics, including number of animal feeding operations, number of WWTPs, and average permitted flow of WWTP effluent, were low. It has been suggested that non-point sources may be responsible for some of these incongruences, especially in watersheds with many livestock feeding operations or fields treated with manure, and there is some evidence that agriculture is a stronger predictor of bass TO occurrence than WWTP effluent [2, 33], but a causal relationship between non-point source EDCs and TOs has

not been established in bass. Overall, the literature suggests that either 1) smallmouth bass can develop TOs in the absence of exposure to estrogenic compounds, or 2), that some locations where estrogenicity was expected to be low, were actually within the range of gonadal sensitivity. However, neither the effects of known exposure to exogenous estrogens, nor the details of normal gonadal development have been examined in smallmouth bass; points that must be considered before TOs can be evaluated as potential biomarkers of estrogenic exposure.

A diverse range of reproductive strategies and pathways of gonadal development are represented even among closely related teleosts. That diversity may be related to the wide ranges of sensitivity thresholds and outcomes of exposure to estrogenic compounds discussed previously. The gonadal phenotype(s) present in individuals of a given species is determined by either genetic or environmental factors, or both. Species are either hermaphroditic, in which both ovaries and testes are present in the same individual (sequentially or simultaneously), or gonochoristic, in which only ovaries or testes are present in an individual. Hermaphroditic species are considered more sexually plastic than gonochoristic species, and the life cycle may involve a transition from a male to a female phenotype under specific conditions [6]. Therefore, hermaphroditic species may be more likely to have stages of development in which intersex gonads are normal, compared with gonochoristic species such as bass and most model fish. But even among gonochorists, there are variations in the specific patterns of gonadal development. Gonochorists follow either the "differentiated" pathway, in which both sexes have primordial, bipotential gonads in early life that later differentiate into either ovaries or

testes (e.g. fathead minnow, medaka, carp, and roach [6, 41-43]); or the "undifferentiated" pathway, in which both sexes initially develop ovaries that later transition into testes in males (e.g. zebrafish [13]). It has been argued that TOs are less likely to occur during the course of normal development in differentiated gonochorists than in undifferentiated gonochorists, which have a transitional ovary-to-testis phase [44]. Therefore, describing the mode of gonadal differentiation in smallmouth bass is an important aspect of evaluating the causes of TO occurrence in wild populations.

The mode of gonadal differentiation and timing of sex-specific cell development has been studied in detail for many gonochoristic fish species. To our knowledge, smallmouth bass gonadal development has not been previously evaluated, but its close relatives, largemouth bass (*Micropterus salmoides*) and bluegill sunfish (*Lepomis macrochirus*) have been reported to be differentiated gonochorists [45, 46]. Therefore, the following generalized overview of the pathway of gonadal differentiation and germ cell development (also summarized in Figure 1) is based on well-studied differentiated gonochoristic species (i.e. medaka [47]; salmonids, *Onchorhynchus spp.* [48, 49]; fathead minnow, [41, 50]; common carp, [43]; and pejerrey, *Odontesthes bonariensis*, [51]).

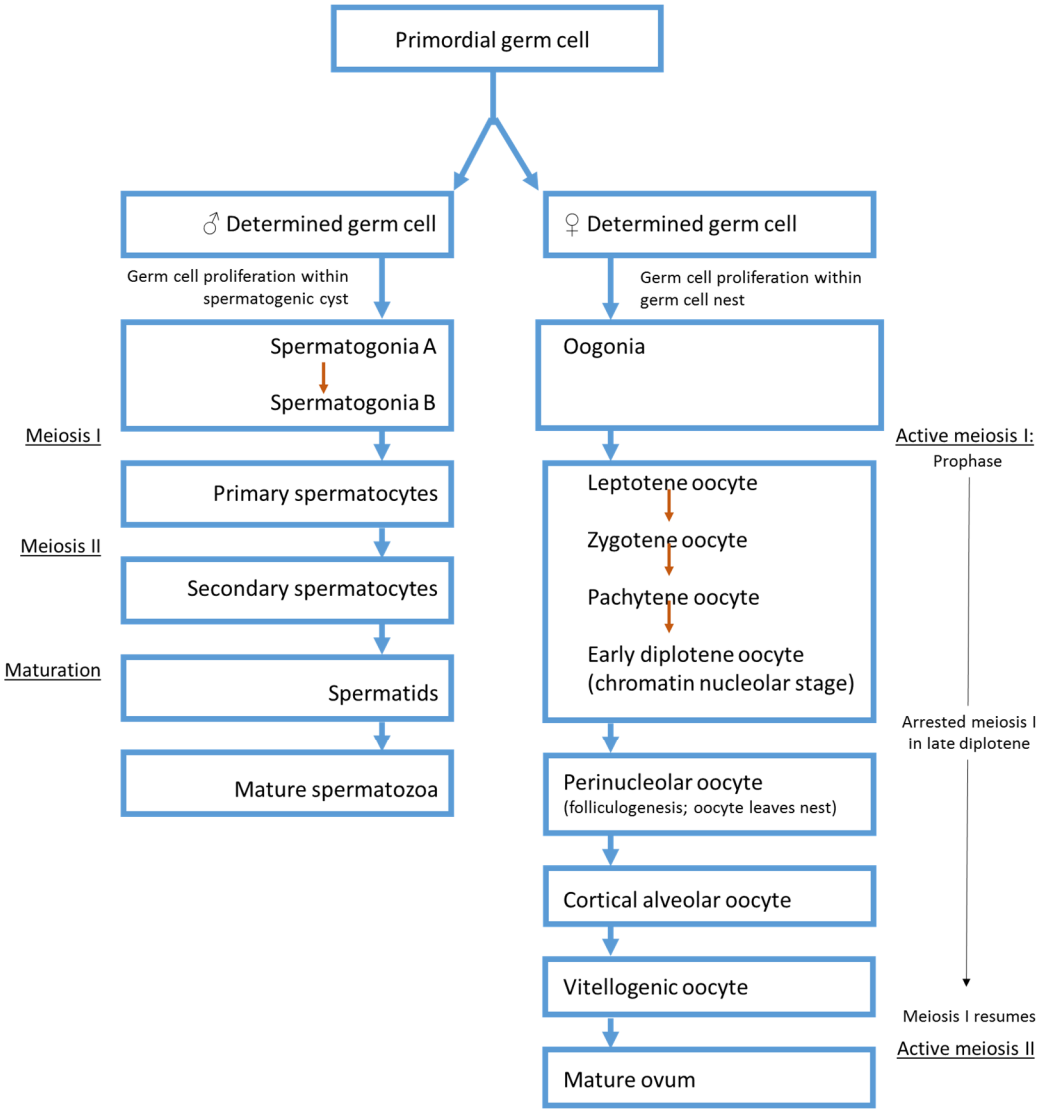
The undifferentiated gonad is composed of somatic cells and a small number of bi-potential, primordial germ cells, which remain quiescent for some days or weeks post hatch [52]. The beginning of differentiation into an ovary or a testis can be observed by the onset of germ cell proliferation, an increase in gonad size, the organization of somatic cells into sex-specific structures (i.e. ovarian cavities or spermatic tubules), the initiation of meiosis, and the differentiation of sex-specific support cells (i.e. granulosa and theca

cells in ovaries; Leydig and Sertoli cells in testes). In some species, the determined sex of the gonad may be identified prior to the onset of differentiation by certain features such as the number of attachments between the gonad and the peritoneum (one in males, two in females), the number of germ cells (greater in females), or by the arrangement of primordial germ cells within the gonad (concentrated near the center in females; scattered throughout the gonad in males). The onset of differentiation usually occurs some weeks or months earlier in females than in males [6, 52].

In ovaries, germ cells differentiate into oogonia, which divide mitotically, resulting in clusters of pre-meiotic oogonia (nests) that are at least partially surrounded by pre-follicle cells and a basement membrane in early stages. Meiosis also begins within the nest, so clusters may be composed of both mitotic oogonia and early-stage meiotic oocytes [49]. Meiosis I arrests in the diplotene stage of prophase, and the oocyte undergoes a period of primary growth prior to entering meiosis II. Early diplotene oocytes, also called chromatin nucleolar oocytes, are identifiable by their distinct "lampbrush" formations in the nucleus [52]. As primary growth of the oocyte proceeds, its size increases rapidly, and it becomes separated from the nest, and surrounded by pre-follicle cells. A single prominent nucleolus appears, followed by multiple nucleoli, which line up around the periphery of the nucleus. In late perinucleolar oocytes, an aggregation of organelles called a Balbiani body forms a spherical or crescent shaped cluster between the nucleus and the exterior of the oocyte. Later still, vacuoles, oil droplets, and cortical alveoli appear. By the end of primary growth, the cortical alveolar oocyte has been completely surrounded by the follicle complex, which includes fully

differentiated granulosa and thecal cells [52]. At the onset of secondary growth, the oocyte accumulates massive amounts of vitellogenin and increases considerably in size [52]. At the end of vitellogensis, secondary growth is complete, and the oocyte enters the second meiotic cycle. At the end of the second metaphase stage, the cell is a mature ovum, and, once released from the follicle during ovulation, is able to be fertilized and complete the end of second meiotic division [52].

Figure 1. Stages of germ cell development in teleosts.



In testes, germ cells differentiate into spermatogonia, which divide mitotically into a cluster of interconnected cells surrounded by Sertoli cells. Subsequent stages of spermatogenesis occur synchronously within this cyst [47]. Spermatogonia undergo two distinct stages of mitosis: type A spermatogonia are larger, and differentiate into type B spermatogonia, which are smaller and present in larger clusters. After the final mitotic division, type B spermatogonia enter the first meiotic cycle and begin the transformation to spermatocytes. Diplotene spermatocytes appear as distinct clusters of very small cells (4-6 µm) with small amounts of cytoplasm, and dense round nuclei. Unlike oocytes, they do not pause for primary growth during the diplotene stage, but proceed to the second meiotic cycle. Following the end of the second meiotic cycle, secondary spermatocytes differentiate into spermatids, which are smaller than spermatocytes, with dense nuclei and indistinct cytoplasm. Spermatids develop into spermatozoa, which are smaller than spermatids, may have faintly visible tails in histologic sections, and are present in the lumen of the spermatic tubule [47].

Relatively few studies of intersex in *Micropterus* have assessed tissue collected from regions where potential influences from both WWTP and agricultural activities are minimal. Thus, there is a clear need to explore the phenomenon in such regions, where known sources of EDCs are minimal, in order to make valid statistical comparisons between populations that are either likely or unlikely to be exposed, and to evaluate the relationship between TO occurrence and human activities that increase likelihood of EDC exposure. The potential impacts of estrogenic compounds on smallmouth bass gonadal tissue can only be adequately assessed within the context of the normal pattern and

timing of gonadal differentiation and development. Likewise, suspected cases of endocrine disruption in wild populations can be better interpreted by studying the effects of exposure to estrogenic compounds in a laboratory setting. Therefore, the goals of this study were, 1) to evaluate TO presence in smallmouth bass populations from watersheds with greater or lesser human impact, 2) to characterize early gonadal development in smallmouth bass in the absence of exogenous estrogens and 3) to characterize the effects of early life exposure to a model estrogen, EE2, on gonadal differentiation.

To accomplish goal 1, the prevalence of adult male smallmouth bass with TOs and the estimated TO density per gonad were quantified for samples collected from eight watersheds with differing degrees of human development. Relationships between TOs and watershed development, human population density, collection season, weight, length, condition factor, age, gonadosomatic index, and hepatosomatic index were assessed. These results are presented in Chapter II.

To accomplish goals 2 and 3, smallmouth bass fry collected from a lake with no known sources of estrogenic compounds were reared in a laboratory, in the absence and presence of the potent model estrogen, 17α-ethinylestradiol (EE2). Standard histological techniques were used to evaluate gonadal tissues in smallmouth bass that were reared in clean water for 180 days or exposed to EE2 (either a low nominal concentration, 2 ng/L, or a high nominal concentration, 10 ng/L) from swim-up to 90 days, followed by 90 days in clean water. Results of that study, presented in Chapter III, include descriptions and images of various stages of ovarian and testicular development ranging from

undifferentiated to fully differentiated stages in control groups and groups exposed to EE2.

A second laboratory study was conducted to evaluate the effects of EE2 concentrations lower than those that caused sex reversal. Yolk-sac stage smallmouth bass were reared in clean water for 120 days, or exposed to 0.1, 0.4, 1.1, or 3.3 ng/L EE2 (nominal concentrations) for 100 days followed by 20 days in clean water. Gonadal tissues were evaluated at 90 days and 120 days, and the histopathological assessment of the proportion of fish that developed ovaries was compared with control groups to determine effects of EE2 exposure. Results of that study are presented in Chapter IV.

II. Testicular oocytes in smallmouth bass in Northeastern Minnesota in relation to presumed exposure to endocrine disrupting compounds

The goal of this study was to assess the testicular oocyte (TO) prevalence and estimated TO density of adult male smallmouth bass collected from eight watersheds categorized as either more or less developed, and therefore either more or less likely to have sources of estrogenic compounds. The strengths of the relationships between TOs and watershed development, watershed human population density, collection season, weight, length, condition factor, age, gonadosomatic index, and hepatosomatic index were evaluated with multiple logistic and linear regressions.

Methods

Collection sites

Collection sites from watersheds with more human development were selected from among lakes and rivers with direct input from municipal wastewater treatment plant (WWTP) effluent. Less developed sites were selected from among lakes with no direct input from WWTP effluent, no agricultural activities, and very little evidence of human development. Other selection criteria included an abundance of smallmouth bass and direct access to the lakeshore via road. The extent of development in each watershed was evaluated with GIS spatial analysis by quantifying human population density, WWTPs, registered feedlots, and percent of agricultural land (crop land and pasture) within the immediate watersheds (the portions draining directly into the sample sites) and the

extended watersheds (i.e. subbasins; areas that drain into the sample site through tributaries). Watershed delineations and land use data were obtained from publically available datasets. Human population data were derived from the US Census Bureau's 2010 census, and apportioned by watershed.

Fish were collected from water bodies in three more developed watersheds and five less developed watersheds (Fig. 2, Table 2). The sites within more developed watersheds included Lake Vermilion (near Tower, MN, St. Louis County), Lake Shagawa (near Ely, MN, St. Louis County), and the lower St. Louis River (near Duluth, MN, St. Louis County). At Lake Vermilion, fish were collected from Pike Bay, located near the small town of Tower (St. Louis County, MN), which has a municipal WWTP that employs stabilization ponds serving roughly 500 households, an immediate watershed human population density of 4 per km^2, and 0.1% agricultural land. The extended watershed of Lake Vermilion is adjacent to the St. Louis River watershed but flows north into the Rainy River basin (Fig. 2), and includes several third order streams and a single fourth order stream (Vermilion River). It has a human population density of 4.0 per km^2, with 0.71% agriculture.

At Lake Shagawa (St. Louis County, MN), fish were collected from points both upstream and downstream of the wastewater treatment plant that serves the town of Ely (1.5 million permitted gallons per day treated with activated sludge, extended aeration, and sand filters [4]). The human population density of the immediate watershed of Lake Shagawa is 80 per km^2 and there is 0.4% agricultural land, compared with 12.3 per km^2 and 0.18% agriculture in the extended watershed. The extended watershed includes

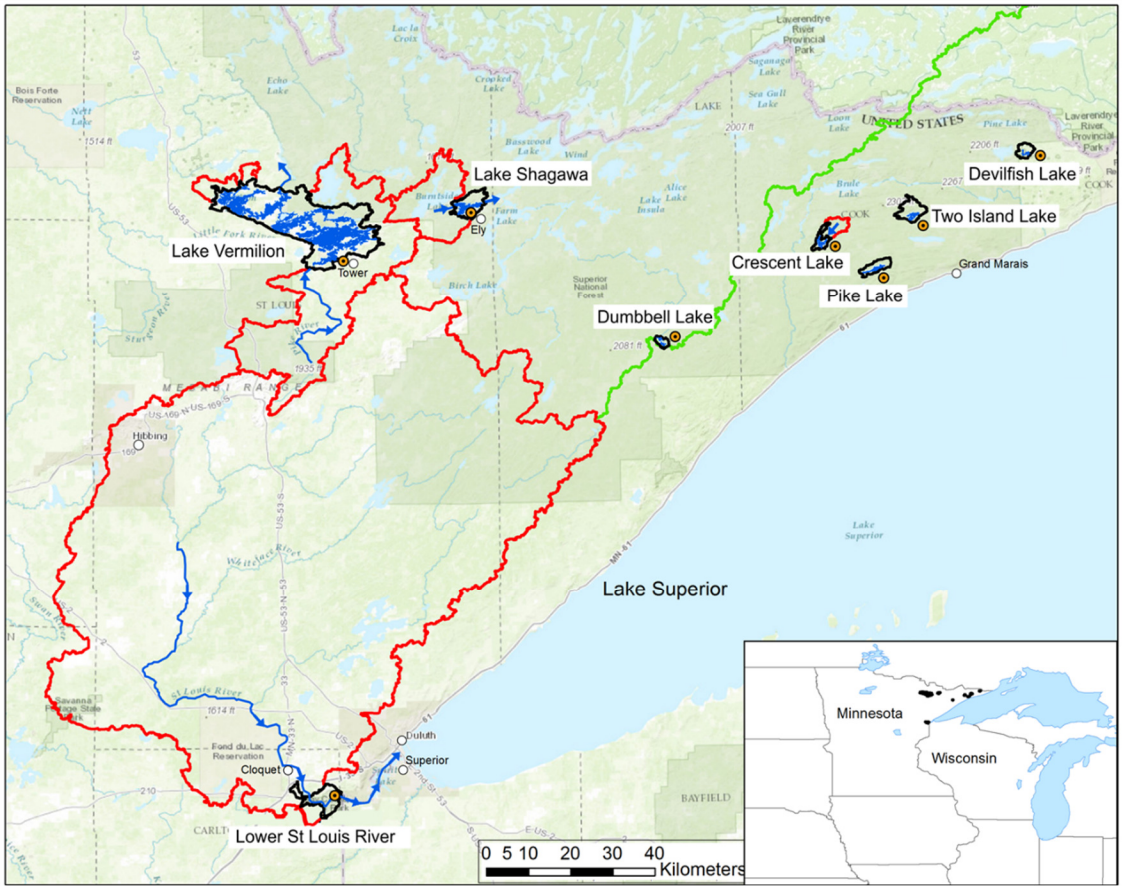
headwaters that enter the west end of Lake Shagawa through a third order stream. The wastewater treatment plant effluent enters the lake on the south shore, and the outflow (Shagawa River) is on the east end of the lake and flows north into the Rainy River basin.

The extended St. Louis River watershed contains 25 minor WWTPs and one major plant, the Western Lake Superior Sanitary District (WLSSD), which has a design flow of 48 million gallons per day, consisting of roughly 50% municipal and 50% industrial waste (mostly pulp and paper mill wastewater [53]), and uses activated sludge, pure oxygen, and sand filters [4]. It has a human population density of 10.9 per km^2, and receives input from municipalities, agriculture, including 3.2% farmed land and 44 feedlots, and current and legacy mining activities associated with the Mesabi Iron Range, including active and restored pit mines, tailings ponds, and residual lean ore piles. The lower St. Louis River watershed, which includes our sampling site, has a human population density of 33 per km^2, 6.8% agricultural land, and has been designated by the International Joint Commission as a Great Lakes Area of Concern because of multiple impairments. The majority of the fish collected from the lower St. Louis River were taken from an area just below the Fond du Lac Dam, located 21 river miles upstream of the Duluth-Superior Harbor and WLSSD outflow, and 9 miles downstream of the city of Cloquet, which conveys its wastewater to WLSSD. Substantial efforts were made to collect fish from a segment of the watershed more directly impacted by WLSSD effluent, but an adequate sample size was not obtained due to poor smallmouth bass habitat.

Sites representing less watershed development included Crescent Lake (12 mi N of Tofte, Cook County, MN), Two Island Lake (12 mi NW of Grand Marais, Cook

County, MN), Dumbbell Lake (4 mi E of Isabella, Lake County, MN), Pike Lake (9 mi W of Grand Marais, Cook County, MN), and Devilfish Lake (12 mi NW of Hovland, Cook County, MN), which all have immediate and extended watersheds with very low human population densities (less than one person per square km) and no WWTP effluent inputs, industrial activities, agricultural land, or feedlots. Two Island, Dumbbell, Pike, and Devilfish Lakes all have immediate watersheds with only first order headwater streams. The Crescent Lake headwaters originate in a small extended watershed that has several second order streams and a single third order stream.

Figure 2. Map of NE Minnesota region where mature male smallmouth bass were collected and evaluated for presence of testicular oocytes (TOs).



Orange circles indicate collection sites within watersheds that were considered either more developed (Lower St. Louis River, Lake Vermilion, and Lake Shagawa) or less developed (Dumbbell Lake, Crescent Lake, Pike, Lake, Two Island Lake, and Devilfish Lake). Immediate watersheds (the portion draining directly into the sample sites) are outlined in black; extended watersheds (i.e. subbasins; area that drains into the sample site through tributaries) are outlined in red. The direction of water flow through the watersheds is indicated with blue arrows. A continental divide is indicated by the green line. Locations of major towns are indicated for reference.

Table 2. Characteristics of the extended and immediate watersheds around sites where smallmouth bass were collected in NE Minnesota

Study Site (ID)	Surface area, km^2	Watershed Area km^2		WWTP[a]		Pop'n / km^2		% Agricultural land		Feedlots	
		Full[b]	Immed.[c]	Full	Immed.	Full	Immed.	Full	Immed.	Full	Immed.
St. Louis River (STL)		9250.24	41.71	25	0	10.9	32.5	3.21	6.80	44	0
Lk. Vermilion (VRM)	158.93	1264.75	387.14	1	1	4.0	3.9	0.71	0.11	0	0
Lk. Shagawa (SHG)	9.49	257.74	34.57	1	1	12.3	80.7	0.18	0.41	0	0
Crescent Lk. (CRT)	3.06	32.09	11.20	0	0	0.1	0.0	0.00	0.00	0	0
Two Island Lk. (TWI)	3.05	24.55	24.55	0	0	0.1	0.1	0.00	0.00	0	0
Dumbbell Lk. (DMB)	1.64	6.11	6.11	0	0	0.5	0.5	0.00	0.00	0	0
Pike Lk. (PIK)	3.30	16.01	16.01	0	0	1.0	1.0	0.00	0.00	0	0
Devilfish Lk.(DVL)	1.64	10.78	10.78	0	0	0.1	0.1	0.00	0.00	0	0

[a] Wastewater treatment plants

[b] Extended watershed, i.e. subbasins; area that drains into the sample site through tributaries

[c] Immediate watershed; area that drains directly into the sample site

Fish collection

Adult male fish greater than 200 mm long were collected just before or during the spring spawning season (surface water temperature 15-21° C) using hook and line or electroshocking methods. In June 2012, fish were collected from the lower St. Louis River, Lake Shagawa, Pike Lake, Two Island Lake, and Crescent Lake. In June 2013, additional fish were collected from the lower St. Louis River and Lake Shagawa, as well as from Lake Vermillion, Dumbbell Lake, and Devilfish Lake. Additional fish were collected during early October 2012 and late September 2013 (surface water temperature 15-20° C) from the lower St. Louis River and Lake Shagawa to examine potential seasonal differences in TO occurrence. We aimed for pooled sample sizes of 100, which provided a 95% likelihood of collecting 10 fish with TOs in a population with 15% prevalence overall [54]. Collection targets were 20 adult male fish per collection site on each visit. Total collections from all visits to a particular site ranged from 21 to 58, resulting in 124 fish from more developed watersheds and 116 fish from less developed watersheds.

Fish were held in aerated or refreshed water containers before being euthanized with an overdose of neutral buffered MS-222 (3 g/L, Western Chemical, Ferndale, WA). Phenotypic sex was determined by gross morphology of the gonads and confirmed by histological evaluation [6]. Total length (to the nearest mm), total weight (to the nearest g), liver weight (to the nearest 0.01 g), and testis weight (to the nearest 0.01 g) were measured. Sagittal otoliths were collected for age determination. Each testis was cut into three roughly equal-sized pieces. Representative transverse sections were taken from the

center of each piece and color-coded with tissue marking dye (Cancer Diagnostics, Inc., Durham, NC) to identify them as anterior, middle, or posterior regions. Tissue samples were placed in Bouin's fixative (Thermo Fisher Scientific, Waltham, MA) for 72 hours, then stored in 10% neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA) until histological processing.

Both sagittal otoliths from each fish were prepared for age determination by fracturing them into two rough halves and stabilizing each piece in modeling clay with the cross section facing up. Annuli were counted in cross sections of three of the four pieces using a light microscope with a small diameter fiber optic light placed directly on the broken edge of the otolith, which was covered with immersion oil [55]. The age of each fish was determined independently by two different researchers, and if readings differed by more than one year, a reading by a third researcher was performed. Age (± one year) was recorded as the reading agreed upon by two out of three researchers, or by the more experienced researcher when only two readings were made.

Histological methods

Testicular tissue was prepared for evaluation using standard histological methods. Briefly, tissues were processed by successive transfers into ethanol and paraffin, and embedded in paraffin blocks. Four μm transverse sections from the anterior, middle, and posterior regions of one testis per fish were collected. Sections were spaced 200 μm apart to avoid capturing the same oocytes in more than one section. Sections were

collected onto glass slides, dried, stained with hematoxylin and eosin, and cover-slipped [56].

Histological sections were evaluated by light microscopy (Zeiss Axiovert 35) and digital images were obtained using a Spot scanner (Diagnostic Instruments) with Spot software. Blazer et al. [32] determined a 90% probability of detecting TOs if 5 sections were examined, even in fish with few TOs. For the present study, six transverse sections per fish were evaluated for the presence of TOs: two each from the anterior, middle, and posterior regions of either the left or right gonad (selected arbitrarily). The number of TOs in each section was counted by scanning at 10x magnification, increasing to 20x if needed to confirm potential TOs. Section area (cm^2) was estimated with the formula $A = \pi ab$, where a is equal to half of the longest aspect of the section and b is equal to half of the shortest aspect of the section, measured to the nearest 0.1 cm. Average TO density (TOs/cm^2) was estimated for each fish by dividing the total number of TOs by the sum of the estimated areas of the six sections evaluated.

Data evaluation

All statistical analyses were conducted using R version 3.2.2 [57]. Two-part mixed effects models were used to determine whether the gonadal region (anterior, middle, or posterior) was correlated with the presence or absence of TOs (modeled with logistic regression) or the magnitude of TO density among fish with TOs (modeled with linear regression). To account for within-site and within-fish similarities, individual fish ID and collection site ID were added as random effects. Models that examined both

logistic and linear effects, logistic only, linear only, and no effects (the null model) were considered statistically equivalent if the Akaike Information Criterion (AIC; [58]) differed by less than 3.8.

Scatterplots of pairs of biometric variables were visually inspected for differences among collection sites. Two-sided t-tests with Hedges g corrections for clustered samples [59] were used to compare the means of age, weight, length, condition factor ([weight, g/total length, cm^3]*100), gonado-somatic index (GSI; [gonad weight, g/body weight, g] *100), and hepato-somatic index (HSI; [liver weight, g/body weight, g] *100) between groups from more and less developed watersheds and between seasons [60]. Differences between groups were considered to be significant at an alpha level of 0.05. The Rao-Scott Cochran-Armitage by Slices (RSCABS) analysis for categorical data was used to test differences in TO prevalence between watershed categories and collection seasons [61].

TO density and probability were evaluated as a function of several characteristics of both landscape (watershed development and human population density in immediate and extended watersheds) and fish (age, weight, length, condition factor, HSI and GSI). Residual plots of linear regressions of TO density vs fish characteristics showed a bimodal pattern such that the samples in which TOs were not detected were grouped separately from the samples in which TOs were detected, suggesting that the data were not normally distributed and that simple linear regression would not be appropriate. Therefore, trends were fitted using two-part models with the likelihood function identified by Duan et al. [62], where the probability of TO occurrence was modeled using

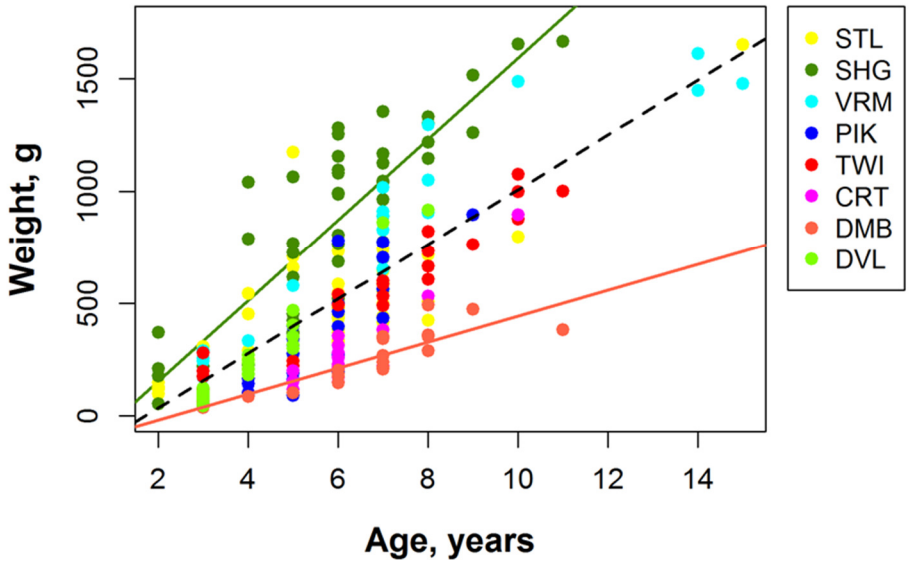
logistic regression, and the magnitude of TO density (among fish with TOs only) was modeled using linear regression. TO density was log transformed for the linear regressions to meet the assumption of normality. A forward selection protocol [63] was followed where variables were added in one at a time, and only retained if they resulted in a significant improvement to the two-part model. The R squared values for the two-part models were calculated according to Nagelkerke [64]. The strength of the relationship between each pair of potential variables was evaluated with correlation analysis. The relative influence of each predictor within a given model was evaluated by rescaling the values of each variable to equal ranges, and comparing the absolute values of the slopes. Three datasets were evaluated separately: 1) the complete dataset of all fish collected in both seasons, where season was both included and excluded from the model selection process, 2) the subset of fish collected during spring only, and 3), the subset of fish collected from sites that were sampled in both spring and fall. Datasets with potential outliers (Cook's distance ≥ 0.5) were re-evaluated after excluding the group containing the outlier. If the removal of the outlier group did not change the outcome of the model significantly, the group was not considered to be an outlier, and results of the analysis of the complete dataset were reported.

Results

Scatterplots of pairs of biometrics indicated slight differences in weights vs. ages among sites; notably, fish from Lake Shagawa (a more developed watershed) were relatively large for their ages, and fish from Dumbbell Lake (a less developed watershed)

were relatively small for their ages (Fig. 3). However, when biometric averages were compared across all fish from more and less developed watersheds (Table 3), very few differences were seen (Appendix A). Mean age within sites ranged from 3.9 to 7.3 years, with no significant difference in mean age between fish from more and less developed watersheds. Mean condition index was higher for fish from more developed watersheds ($p = 0.030$) than from less developed watersheds when spring and fall data were combined. Among spring-collected fish, there was no significant difference in condition index between more and less developed watersheds, but both weight and length were higher among fish from more developed watersheds ($p = 0.042$ and $p = 0.025$, respectively). There were no significant differences in mean HSI or GSI between fish from more and less developed watersheds.

Figure 3. Scatterplot of ages and weights of male smallmouth bass collected from eight sites in NE Minnesota.



The dashed line represents the overall trend among all fish collected; the green line represents fish collected from Lake Shagawa (SHG), which were larger than average for their age; the orange line represents fish from Dumbbell Lake (DMB), which were smaller than average for their age.

Table 3. Summary of biometrics and testicular oocytes in smallmouth bass collected from NE Minnesota waters considered either more developed or less developed. Presented as mean (standard error).

Study Site (ID)	*N*	Age[a]	Weight[b]	Length[c]	Condition Index[d]	GSI[e]	HSI[f]	TO Prev.[g]	Avg. TO Density[h]
St. Louis River (STL)	58	5.7 (0.3)	444 (33)	312 (7)	1.4 (0.0)	0.7 (0.0)	1.0 (0.0)	53%	4.21 (1.26)
Lk. Vermilion (VRM)	20	7.3 (0.8)	831 (103)	371 (17)	1.4 (0.0)	1.0 (0.1)	1.3 (0.1)	55%	2.28 (1.52)
Lk. Shagawa (SHG)	46	5.9 (0.3)	845 (64)	366 (11)	1.5 (0.0)	0.9 (0.0)	1.4 (0.1)	59%	2.22 (0.67)
Avg, more developed:	**124**	**6.0 (0.2)**	**656 (37)**	**353 (6)**	**1.4 (0.0)**	**0.8 (0.0)**	**1.2 (0.0)**	**55%**	**3.16 (0.69)**
Crescent Lk. (CRT)	21	6.3 (0.2)	291 (37)	274 (9)	1.3 (0.0)	0.7 (0.0)	1.2 (0.1)	43%	1.03 (0.34)
Two Island Lk. (TWI)	21	6.9 (0.5)	573 (63)	327 (19)	1.5 (0.0)	0.6 (0.0)	1.2 (0.1)	38%	1.22 (0.6)
Dumbbell Lk. (DMB)	21	6.5 (0.5)	240 (29)	258 (12)	1.2 (0.0)	1.0 (0.1)	1.4 (0.1)	19%	0.25 (0.18)
Pike Lk. (PIK)	24	5.6 (0.3)	359 (49)	282 (13)	1.4 (0.0)	1.0 (0.1)	1.2 (0.1)	8%	0.11 (0.09)
Devilfish Lk.(DVL)	29	3.9 (0.2)	231 (40)	246 (12)	1.2 (0.0)	0.7 (0.0)	1.6 (0.1)	7%	0.07 (0.05)
Avg, less developed:	**116**	**5.7 (0.2)**	**332 (23)**	**276 (6)**	**1.3 (0.0)**	**0.8 (0.0)**	**1.3 (0.0)**	**22%**	**0.49 (0.14)**

[a] Determined by sagittal otolith annuli, ± 1 year

[b] Wet weight, g

[c] Total length, mm

[d] Condition index (body weight, g/total length, cm^3)*100

[e] Gonado-somatic index (gonad weight, g/body weight, g) *100

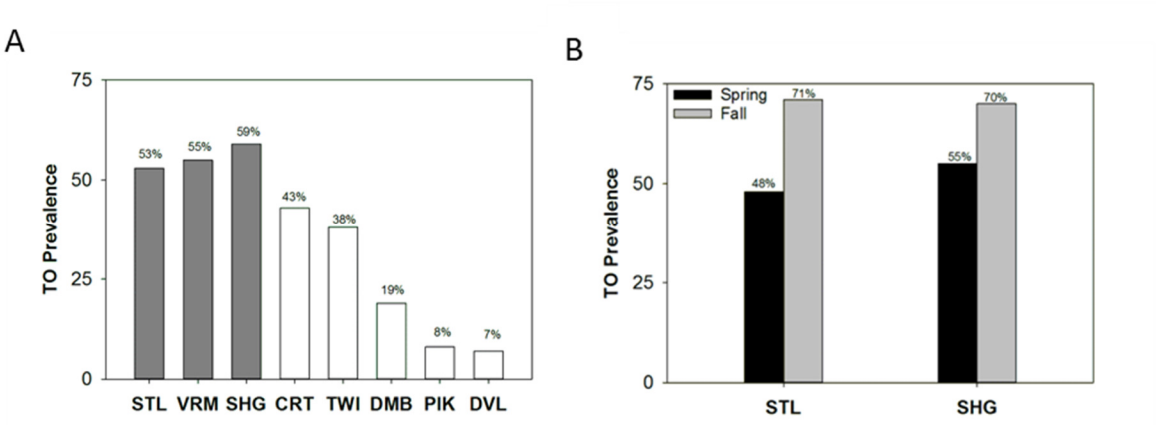
[f] Hepato-somatic index (liver weight, g/body weight, g)*100

[g] Percent of males with testicular oocytes

[h] Average number of TOs per cm^2 of tissue section evaluated

In the complete dataset of all male fish collected in both spring and fall, mean TO prevalence in more developed watersheds was 55% (range = 53-59%, n = 3 sites) and mean TO density was 3.16/cm^2 (Fig. 4A). Male fish from less developed watersheds had a significantly lower mean TO prevalence (22%; $p < 0.001$), but site prevalence varied more widely (range = 7-43%, n = 5 sites) than in more developed watersheds. TO density averaged 0.49/cm^2 in fish from less developed watersheds (Fig 4A). Among fish collected from more developed watersheds in the spring only, mean TO prevalence was also significantly higher (51%; $p = 0.002$) than less developed watersheds (22%). Among fish collected from two sites in more developed watersheds in the fall, mean TO prevalence was 71%, and mean TO density was 8.2 per cm^2. Fish collected from the same two sites in the spring had lower mean TO prevalence (51%, $p = 0.045$) and density (1.9 per cm^2; Fig 4B).

Figure 4. TO prevalence among male smallmouth bass collected from eight sites in NE Minnesota.



Percent of mature males with at least one TO observed among 6 transverse sections is shown for the complete dataset of all fish collected during both spring and fall from either more developed (shaded bars) or less developed (white bars) watersheds (A), and fish collected from two more developed sites during both spring (black bars) and fall (shaded bars; B).

In statistically equivalent models for the complete dataset of all fish collected in both spring and fall, more human development in the watershed was identified as one of two parameters that predicted TO probability (Fig 5A), with the second as either HSI (Fig. 5B), age (Fig. 5C), weight, or length. HSI was negatively correlated with TO probability, while age, weight, and length were positively correlated. More human development was also identified as one of the two parameters predicting the magnitude of TO density in fish with TOs in the complete dataset (Fig. 5D), with the second parameter as either weight (negatively correlated; Fig. 5F) or human population density within the extended watershed (positively correlated; Fig. 5E). Including season as a potential parameter did not improve the models; therefore, it was excluded from the model selection procedure. Fit of the data was poor, with R^2 ranging from 0.19 to 0.21 in all statistically equivalent models.

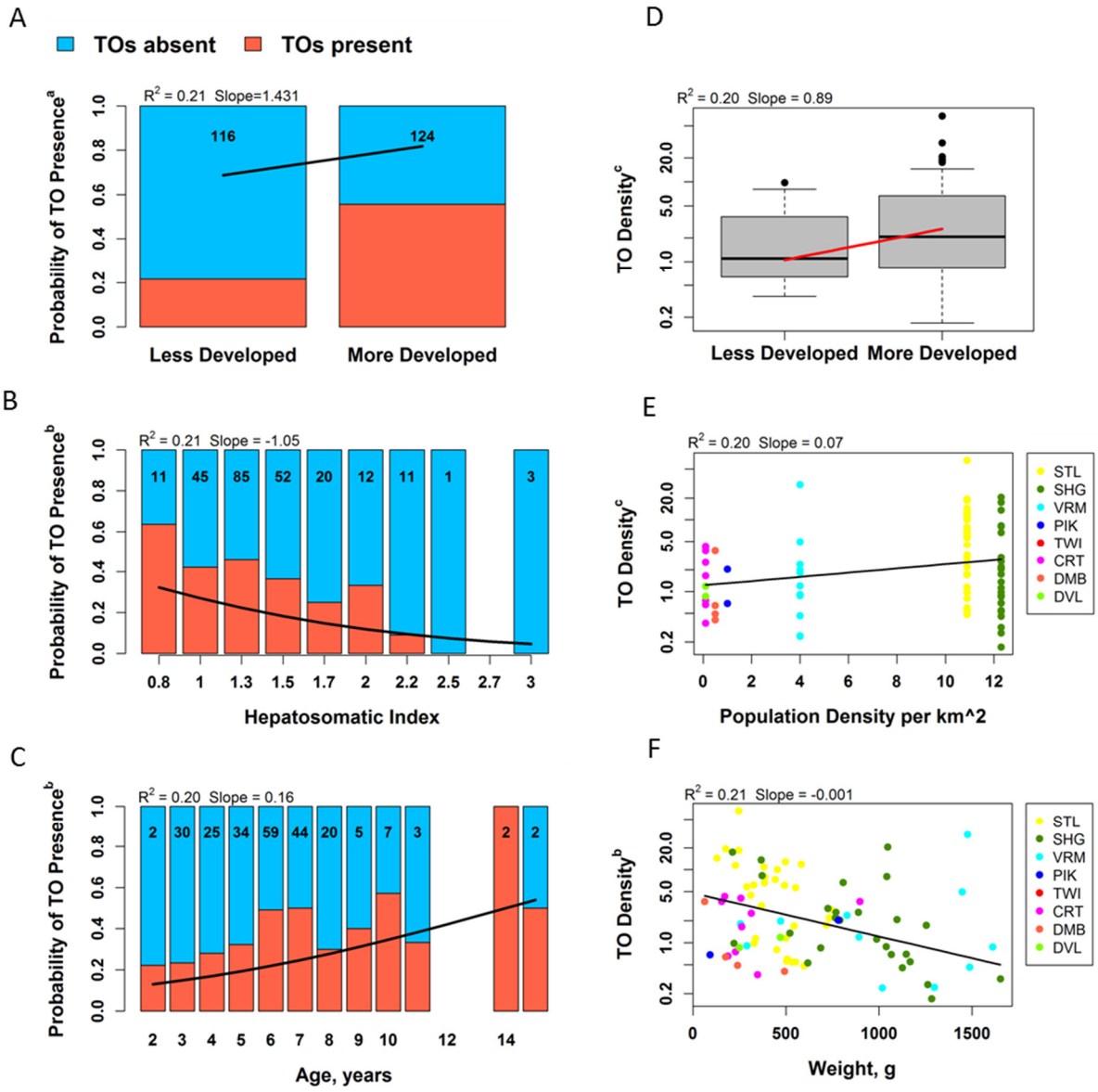
Models developed from the subset of fish collected only in spring showed generally similar patterns. In all statistically equivalent models for this dataset, the probability of TO presence was positively correlated with watershed development plus either age or length, and the magnitude of TO density was significantly correlated with each of the other variables individually, although the fit of the data was poorer than that of the full dataset (R^2 = 0.13 to 0.15).

For the subset of data from sites at which fish were collected during both spring and fall (St. Louis River and Lake Shagawa), the probability of TO presence was predicted by HSI, and the magnitude of TO density among fish with TOs was predicted either by weight or length in statistically equivalent models (R^2 = 0.23 to 0.25).

Therefore, the outcomes of the models for each data subset were consistent with those from the complete dataset, suggesting that the predictors of TOs were similar among fish regardless of collection season.

Figure 5. Relationships between watershed and biometric characteristics and TOs in smallmouth bass

The probability of TO presence was correlated with watershed development category **(A),** hepato-somatic index **(B),** and age **(C)**. TO density among fish with TOs was correlated with watershed development category **(D),** watershed population density **(E)**, and fish weight **(F)**.



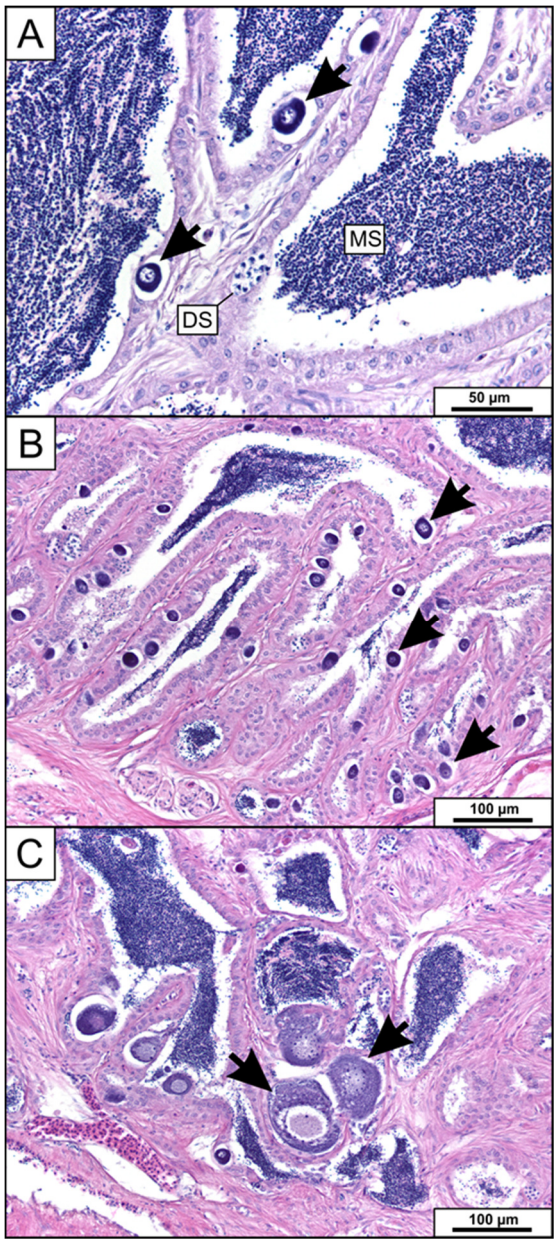
Numbers on bars represent the total number of fish in that category. Each graph represents one variable in a multiple regression, with the variable not shown on the x-axis treated as indicated by

superscripts: [a] Hepatosomatic index = mean HSI; [b] Watershed development category = average effect of development category; [c] Weight = mean weight, g

All testicular oocytes observed in this study were primary (pre-vitellogenic) oocytes that were usually embedded in the germinal epithelium of spermatic tubules (Fig. 6A), but occasionally appeared as free-floating cells within the tubule lumen. TOs were typically located near the central region of the testis cross-section, close to a major blood vessel. Less frequently, TOs were found around the perimeter. Most fish with TOs only had a few scattered oocytes in each testis cross-section, although rarely, several dozen oocytes appeared across more than one field of view (1.0 mm at 20x mag.; Fig. 6B). TOs were almost always in an early stage (chromatin nucleolar), measuring approximately 15-20 μm in diameter, with densely stained cytoplasm and a clearly defined nucleus. However, in two individuals from Lake Vermillion (a more developed site), at least one TO was in a late stage of primary growth (perinucleolar stage) with larger diameter (35-100 μm), nuclei containing multiple nucleoli around the periphery, and small vacuoles in the cytoplasm (Fig. 6C).

On the basis of histological examination, all testes with oocytes appeared to be undergoing normal spermatogenesis with no indication of impaired reproductive function or feminized structure. In tissues that were most extensively affected by TOs, the affected region still had a predominantly male phenotype, rather than appearing as a discrete section of ovarian tissue within the testis (Fig. 6B). Germ cell degeneration, fibrosis, granulomatous inflammation, or parasites were occasionally observed, but did not appear to coincide with presence of TOs.

Figure 6. Photomicrographs of testicular tissue of smallmouth bass (*M. dolomieu*) collected from NE Minnesota.



Testicular oocytes (TOs; arrows) appeared most commonly as scattered chromatin nucleolar stage oocytes (**A**) in the germinal epithelium of spermatic tubules with developing spermatocysts (DS) and mature spermatozoa (MS). Rarely, numerous TOs appeared in a more extended region of testicular tissue (**B**), or as perinucleolar stage oocytes (**C**). H&E stain.

The mean TO density among sections collected from the anterior, middle, and posterior regions of the gonads were 1.7, 1.1, and 3.1 TOs per cm^2, respectively, with wide ranges (Appendix B). The mixed effect model results suggested that there were significant effects of section location on both TO presence and density. Specifically, sections collected from the middle region of the gonad had 72% lower odds of containing TOs than the anterior or posterior regions ($p = 0.002$), and sections collected from the posterior region of the gonad had 1.8 times higher TO density than the anterior portion ($p < 0.05$). There was no significant difference in TO density between the middle and anterior regions.

Conclusions

These data, showing smallmouth bass testicular oocyte prevalence ranging from 7-59% in NE Minnesota, are consistent with reports of TO prevalence in bass populations in widespread parts of North America [23]. By analyzing testicular tissue from mature male smallmouth bass living in watersheds with varying amounts of human development, we concluded that human activities, and by proxy, the likelihood of fish exposure to estrogenic compounds, were only weakly related to the presence and extent of TO development. While mean TO prevalence was higher in bass populations from more developed watersheds (55%) compared to those from less developed watersheds (22%), it was not consistently low in populations from less developed watersheds, as would be expected if estrogenic compounds or other human impacts were a unique cause. Mean TO density showed a similar pattern; slightly higher in populations from more developed

watersheds (3.16 per cm^2) compared to those from less developed watersheds overall (0.49 per cm^2), but not consistently low in this latter set.

The large number of parameters identified in statistically equivalent models can be partly explained by correlation (e.g. r = 0.95 between length and weight), but might also reflect the poor fit of the models (R^2 = 0.19 to 0.21). These results also suggest that, while TOs were correlated with land-use factors that increase the probability of fish exposure to estrogenic compounds, they were also, and often more strongly, correlated with other factors with no obvious relationship to estrogenic compounds. For instance, while watershed development and human population density were associated with the probability of TO presence, the correlations between TO presence and fish age, length, and HSI were stronger. Similarly, fish weight was more strongly associated with TO density than either watershed development or human population density. Our size and weight data are consistent with the observation that primary productivity, and therefore fish growth rate, may have been higher in more developed watersheds than in less developed watersheds, which could explain the association of weight with TO occurrence. Corresponding with this theory, TO prevalence in largemouth bass (*M. salmoides*) from Georgia impoundments with very little influence from agriculture or municipal wastewater was negatively correlated with surface area, and researchers noted that smaller impoundments were more eutrophic than larger ones, although they did examine the correlation between TO prevalence and fish size [40].

The likelihood of EDC exposure in NE Minnesota could also be influenced by chemical, hydrologic or biological factors beyond the scope of this study. These might

include unknown sources of estrogenic compounds that were not predicted by land use, or by water retention time, flow direction, primary productivity, population genetics, behavior, or the extent of an individual fish's home range relative to presumed sources of estrogenic compounds. Overall, our results provide little support for a dose-response pattern between likely exposures to estrogenic compounds and TOs, and suggest that other unknown factors may influence smallmouth bass TOs. In contrast, an exposure-response pattern has been demonstrated between WWTP discharges and intersex in roach in the UK and darters in Canada, suggesting a causal relationship [28, 31, 65, 66].

Our observations that TOs were most frequently found in the chromatin nuclear stage, and only rarely in the perinucleolar stage, are consistent with other data from *Micropterus* [32, 35]. Histologically, the predominance of normal testicular tissue in gonads from individuals with even the most extensive TO development does not suggest that TOs are hindering spermatogenesis, but no functional measures of sperm quality were made. Laboratory studies have demonstrated that sperm production or quality can become impaired in some species (e.g. roach, zebrafish) with induced feminization of testicular tissue [65, 67], but there are few data on the relationship between intersex and fertility in wild fish. Blazer et al. [35] demonstrated a significant inverse relationship between sperm motility and TOs in wild smallmouth bass. In that study, fish collected from a site with low likelihood of EDC exposure had a higher percentage of motile sperm (86%) than fish from sites with higher exposure likelihood (17-29% motility) as well as lower TO prevalence (11% compared with up to 50-100%), although there was no difference in sperm viability among sites.

We detected an increased likelihood of TO development in the posterior region of the gonad compared with the middle and anterior regions. Although this may be a minor effect with unknown biological relevance, it indicates that attention should be paid to sampling techniques, and may suggest a need for developing a common set of sampling practices among researchers in this field. Similarly, the increased TO occurrence and density in fall-collected fish is noteworthy, but not consistent with other studies, which have reported either no difference between spring and fall TO prevalence or a decrease in fall TO prevalence compared with spring [23, 35]. Seasonal differences in TO prevalence could be important to future investigations into the mechanism by which TOs develop, given that the state of the gonad and its cellular processes are different at the end of the reproductive season compared with the beginning. Many of the testicular tissue samples collected during the fall season appeared to be undergoing recrudescence, in which the germinal epithelium is rebuilt via proliferation and differentiation of germ cell precursors. It may be speculated that this period of increased differentiation leads to an enhanced likelihood of TO development, whether they are caused by some exogenous stimulus or develop spontaneously.

An additional outcome of this work is the development of an alternative method for estimating the extent to which fish are affected by TOs. The large size of mature *Micropterus* gonads makes it infeasible to serially section the entire organ; therefore, the presence of TOs must be estimated by some sampling procedure. Most other studies of *Micropterus* TOs have employed a severity grading system, which is a well-established approach to histopathological evaluation [32, 56, 68]. The TO density estimation method

developed for the present study is similar to severity grading in that it assumes the existence of a dose-response relationship, where increased exposure to some stimulus results in more extensive TO development, although there are several key differences compared with the most commonly used severity index for *Micropterus* TOs developed by Blazer et al. [32]. That method assigns a grade from 0 to 4 based on only the most extensively affected field of view (approximately 4 mm^2), which is fast and simple, but introduces a sampling bias, in that the grade is based on a larger proportion of tissue in small gonads compared with large gonads. In contrast, the TO density method avoids gonad-size bias by normalizing the total number of TOs by the area of the entire section. Additionally, where the Blazer index assigns a higher severity grade to sections with clustered TOs than to sections with diffusely distributed TOs, our TO density estimate does not presume that clusters of TOs represent a more severe condition. Severity indexes developed for other species (i.e. European flounder, *Platichthys flesus*, and roach [24, 68]), also consider TO distribution, but to our knowledge, there is no evidence that clustered TOs represent a higher likelihood of adverse outcome (e.g. reduced reproductive output) compared with diffusely distributed TOs. A separate, but related issue is that the severity levels in existing grading systems are arbitrary categories without continuous quantitative basis, which means that averaging the grades of many sections from a single individual or the grades of many individuals from a single collection site is statistically unsound [61]. As a continuous variable, the TO density estimate can be analyzed with statistical methods that are inappropriate for categorical indexes. A drawback is that the TO density estimation is more time-consuming than the

Blazer severity index. It is also somewhat dependent on the requirement that the TOs are of similar size across all samples, an assumption that seems reasonable in this dataset for all but two fish, in which large, late stage primary oocytes were observed (Fig 6C).

III. Characterization of early gonadal development in smallmouth bass (*Micropterus dolomieu*) and effects of 17α-ethinylestradiol on sex differentiation

This chapter describes the results of an experiment to characterize the stages of gonadal sex differentiation in male and female smallmouth bass reared in clean water for 180 days, and to evaluate the effects of EE2 exposure on gonadal development. Because the sensitivity of this species to EE2 was unknown, the exposure concentrations (2 ng/L and 10 ng/L) were selected to serve as range-finders, rather than to provide specific effect concentrations. Potential outcomes of particular interest included the identification of the earliest stage of development at which TOs could be diagnosed, if present, and the occurrence of TOs in control and EE2-exposed fish.

Methods

Collection of wild smallmouth bass

Pike Lake (9 mi W of Grand Marais, Cook County, MN) was selected for the collection of wild smallmouth bass fry, from among the many remote lakes in NE MN, because of its good water clarity, accessibility of nests, and low likelihood of fish exposure to estrogenic compounds, due to the absence of wastewater treatment plants and agricultural activities, minimal human development along the lakeshore, and a low human population in the immediate watershed (<1 person/ km^2; Chapter II). We previously reported a relatively low prevalence of testicular oocytes in mature male bass

collected from Pike Lake (8%, n = 24) compared with others in the region (range = 7% – 59%; Chapter II). For the present study, fish collections were performed approximately at the peak of spawning season, by SCUBA divers with large bore syringes ("slurp guns", Florida Keys Watersports, FL). Approximately 400 fry were collected from each of 4 nests with actively guarding males and large clutches (several thousand) of yolk-sac fry. The age of fry at the time of collection appeared be less than 7 days post hatch, based on low mobility and the presence of yolk sacs, although slight differences in yolk sac size, and therefore presumed age, were observed among the four nest cohorts. Fry were placed in 1 L Nalgene bottles with native lake water (20.9° C), and headspaces were filled with pure oxygen. Bottles were tightly packed into an insulated container to limit temperature fluctuations and mechanical agitation during transportation. During the four hours that elapsed between collecting fry and transferring them to exposure tanks in the laboratory, dissolved oxygen was intermittently monitored to ensure that it remained above 60% saturation.

EE2 exposure stock preparation

EE2 stock bottles were prepared by a shell-coating method, where 100 µL of 733.4 mg/L EE2 superstock (in 100% ethanol; Sigma-Aldrich) was added to a 1 L solvent cleaned glass flask. The flask was rotated while filtered air was introduced to evaporate the solvent, evenly coating the inside of the flask with EE2. The flask was then covered with aluminum foil and stored at 4º C until needed. Every 7 days, a shell-coated flask was filled with 1 L of deionized water and stirred for 24 hours. The contents of the

1 L flask were rinsed into a glass carboy with 18 L of filtered Lake Superior water (LSW), resulting in 19 L of EE2 stock (3860 ng/L nominal).

Experimental design and exposure conditions

A flow-through exposure system at the US EPA Mid-Continent Ecology Division (Duluth, MN) was calibrated to dilute EE2 stock to nominal concentrations of 10 and 2 ng/L EE2 in LSW using small-displacement volume dual-head ceramic piston pumps (Fluid Metering, Inc.; FMI; Syosset, NY) supplying stainless steel mixing vessels. Correct dilution ratios were confirmed volumetrically. Exposure and control waters were delivered to exposure chambers with peristaltic pumps via Teflon tubing. The exposure chambers consisted of preassembled glass aquaria with silicone-sealed joints, clear acrylic covers, and stainless steel standpipes positioned to allow a filled volume of 16 L. Chambers were randomly placed in an enclosed, circulating water bath. The exposure system was in operation for 1 week prior to introduction of the test organisms in order to equilibrate all surfaces with EE2.

To control for the apparent differences in age at the time of collection, as well as to promote even growth rates and minimize aggression within each tank, a randomized block design was used. Each of the three experimental treatments (control, low, and high EE2) consisted of four replicate exposure tanks, each of which was loaded with 100 arbitrarily selected fish from one of the four nest cohorts.

Incoming water was tempered to 22.3° C using electronically controlled mixing valves with an alarm threshold of ±1° C. Temperature was measured daily in at least one

tank per treatment. The flow-through rate was initially set at 12 volume additions per 24 hours (140 mL/min) and increased to 280 mL/min as biomass increased, to reduce loading below 0.5 g per 1 L flowing through in 24 hours, as recommended by ASTM guidelines for early life-stage tests [69]. Tanks were supplied with filtered air through disposable borosilicate glass Pasteur pipettes. If oxygen saturation fell below 60% in any tank, aeration and/or flow rate was increased until all tanks measured >60% oxygen saturation. pH was measured weekly in at least 1 tank per treatment. Hardness, alkalinity, and conductivity of incoming LSW measured 44 mg $CaCO_3$/L, 45 mg $CaCO_3$/L, and 102.6 µS, respectively, at the beginning of the exposure. LSW has little variability in these parameters, and they were monitored frequently by other researchers in the facility during the experiment. Total ammonia was measured in each tank four times (days 24, 49, 117, and 179) with an ion-specific electrode. The light cycle was maintained at 16:8 light:dark throughout the experiment.

Fish from two of the four nest cohorts began to swim up and feed on test day 0; fish from a third cohort swam up on day 1, and fish from the final cohort swam up on day 2. Fish were fed live brine shrimp nauplii (*Artemia spp.*) two or three times daily from swim-up until test day 21, when blackworms (*Lumbriculus spp.*, California Blackworm Co., Fresno, CA) were added to the diet. Between test days 21-89, the *Artemia* ration was gradually decreased and replaced with *Lumbriculus*, until the majority of fish in each tank were able to consume their daily ration in a single feeding of *Lumbriculus* (Table 4). Between days 126 and 170 the *Lumbriculus* diet was supplemented with earthworms (*Eisenia fetida*; Uncle Jim's Worm Farm, Spring Grove, PA) due to a supply shortage of

Lumbriculus. Prior to day 15, ration was not fixed, and fish were fed to excess. Beginning on day 15, delivered rations were estimated on a wet weight: wet weight basis. Delivered rations were initially high to encourage fast, even growth rates within tanks, and gradually decreased throughout the experiment, from an average of 46% of fish wet weight between days 15 and 40; to 24% between days 40 and 92, and 14% between days 92 and 179 (Table 4). Consumption rates were not estimated, but were certainly lower than the delivered rations while *Artemia* was provided, and while fish were transitioning to *Lumbriculus,* as indicated by uneaten food that was removed during cleaning. During the period when the diet was composed of only *Lumbriculus* (starting on day 90), leftover worms were rarely observed; therefore, consumption rates were similar to delivered rations.

Table 4. Growth and ration data for smallmouth bass fry reared for 180 days.

Test day	N per tank	Avg wet weight (SD), g	Avg total length (SD), mm	Avg daily ration wwt:wwt	Diet composition
0	100	0.01 (0.00)		ad lib.	100% BS
6	97	0.03 (0.00)	13 (1)		
15	93	0.09 (0.02)	20 (1)	49%	89% BS 11% LV
25	90	0.16 (0.02)	25 (1)	45%	76% BS 24% LV
32	60	0.20 (0.03)	28 (2)	38%	55% BS 45% LV
40	57	0.23 (0.04)	29 (2)	34%	23% BS 77% LV
49	54	0.30 (0.02)	32 (3)	25%	100% LV
63	44	0.54 (0.19)	38 (5)	21%	100% LV
74	35	0.99 (0.51)	44 (8)	19%	100% LV
92	21	2.3 (0.37)	57 (9)	23%	100% LV
120	16	6.9 (1.7)	83 (7)	11%	89% LV 11% EF
151	10	11.7 (2.8)	102 (8)	9%	75% LV 25% EF
180	8	18.4 (4.0)	118 (8)		

Abbreviations: BS, live brine shrimp nauplii (*Artemia spp*); LV, live blackworms (*Lumbriculus spp*); EF, live earthworms, *Eisenia fetida*

As tank biomass increased, fish were removed to reduce loading below 5 g of biomass per 1 L of standing volume, per ASTM guidelines [69]. On day 126, half of the fish from each tank were transferred into a second set of tanks (increasing the number of replicates from 4 to 8 per treatment) to allow for continued growth without overcrowding.

Tanks were cleaned daily by scrubbing and siphoning debris and uneaten food. A sterilization procedure was performed every two weeks during the exposure period (test days 0 through 90) to prevent a buildup of microbial biofilm that might reduce EE2 concentrations during a long-term experiment. First, all fish were transferred by dip nets into a duplicate set of exposure tanks fed by a redundant set of EE2-delivery pumps and lines, providing uninterrupted exposure. A 4.7% solution of peracetic/acetic acid sterilant (Mar Cor Purification, Plymouth, MN) was delivered through the vacated tanks, pumps, and lines and circulated for 10 minutes before flushing with clean LSW. The clean tanks were left dry until prepared for the next sterilization.

EE2 analysis

Analyses of tank water samples were conducted to confirm the qualitative characterization of each exposure treatment as either control, low (2 ng/L EE2 nominal), or high (10 ng/L EE2 nominal). Exposure tank samples were collected at 7 time points just before the biweekly sterilization procedure and at 5 time points just after sterilization. EE2 stock bottle samples were collected at 7 time points. Samples were capped, stored at 4 ºC, and analyzed within 24 hours. Tank samples, blanks, and matrix spikes were concentrated to within the range of analytical standards (50 – 3000 ng/L) using solid

phase extraction (SPE) columns (JT Baker, C18, 500 mg). Stock bottle samples were diluted 10x for analysis. EE2 concentrations were measured with enzyme-linked immunosorbant assay (ELISA) kits (Abraxis Ecologenia ethynyl-estradiol 96-well microplate, Warminster, PA), using the recommended procedures, with a stated method detection limit of 1 ng/L prior to concentration. Briefly, antigen-enzyme conjugate solution and EE2 sample or standard were added to duplicate wells to initiate a concentration-dependent competitive reaction with antibody binding sites coating the wells. After a 60 minute incubation, unbound EE2-enzyme conjugates were removed with a wash solution. A colorimetric reaction was initiated with a substrate that is converted to a colored product in the presence of EE2-bound antibodies, and then terminated with a stop solution. Absorbance at 450 nm was fitted to a 4-parameter logistic regression (SigmaPlot 12.0) to produce a standard curve. Sample concentrations were calculated by interpolation using the standard curve produced at the same time as each set of samples. Measured EE2 concentrations for each exposure treatment were reported as the mean and standard deviation of all samples analyzed.

Histological sample preparation

On days 25, 32, 40, 49, 63, 74, 92, 120, 151, 165, and 180, subsamples of 3-12 fish were collected from each exposure chamber and prepared for histological evaluation. Fish were randomly selected and euthanized with an overdose of MS-222 (3 g/L, Western Chemical, Ferndale, WA, buffered to neutral pH with $NaCO_3$). Total lengths and wet weights were recorded. Fish less than 1 g wet weight were placed whole into Bouin's

fixative, while those larger than 1 g (test days 74-180), were slit ventrally and abdominal cavities and gill cavities and were perfused with Bouin's fixative. After 72-hours the samples were transferred to neutral buffered formalin for storage until processing.

Prior to histological processing, fish were trimmed down to the abdominal portion between the vent and posterior edge of the gill chamber, and in larger fish (test days 150, 165, and 180), gonads and dorsal peritoneum were excised from the abdominal chamber. Tissues were then dehydrated in ethanol, infused with paraffin, and embedded in blocks. A preliminary evaluation of both transverse and longitudinal sections of gonads from day 165 fish showed that the anterior-most regions were not consistently representative of the most advanced stage of development; therefore, a sectioning protocol was developed that focused on transverse sections of the middle and posterior regions. Each trunk piece or gonad pair with its associated tissue was bisected transversely through the mid-posterior region and both halves were embedded, cut-face down, in a single paraffin block. For each fish, six sections (4 μm thick) of fully faced gonad cross-sections, spaced 50 or 100 μm apart, were collected, stained with hematoxylin and eosin, and evaluated by light microscopy. For each fish, the most advanced stages of germ cells and somatic structure observed among the sections were reported. The criteria used to identify the gonad phenotype of each fish as undifferentiated, presumptive ovary, definitive ovary, or testis, were developed during a thorough initial evaluation of samples at each collection point. The largest-observed diameter of germ cells, blood vessels, and gonad cross sections were measured to the nearest μm with a light microscope (Zeiss Axiovert 35) calibrated with a stage micrometer using a Spot scanner (Diagnostic Instruments) with Spot

software. Measurements were reported as the range of maximum measurements observed in each group. Deviations from the control group, in terms of germ cell stages, somatic structure, and proportion of fish that developed ovaries, were used to determine EE2-exposure-related effects on gonadal development throughout the exposure and grow-out periods.

Statistical analysis

All statistical analyses were performed with R version 3.2.2 [57]. Differences between groups were considered to be significant at an alpha level of 0.05. Welch's t-test was used to compare the mean measured concentrations of EE2 in experimental tanks before and after the biweekly sterilization procedure, in order to evaluate the stability of the toxicant in the system over time. Fish wet weights were log-transformed to meet the assumption of normality. Dunnett's multiple comparison test was used to determine whether mean fish total length and mean wet weight differed between the treatments and the control in each age group. Chi-square analysis was used to detect significant deviations from the expected proportion of fish that developed normal ovaries (0.5) for age groups in which both ovarian and testicular differentiation was evident in controls (days 92, 120, 151, 165, and 180).

Results

Water quality measurements

Temperature and pH remained consistent in all treatments throughout the experiment, with means (± SD) of 22.3 (± 0.3) ° C and 7.6 (± 0.2), respectively. Dissolved oxygen measured >60% saturation in all tanks on most days, and was not <45% in any tank for longer than 24 hours. Overall mean (± SD) dissolved oxygen was 6.8 (± 0.8) mg/L, corresponding to 78 ± 11% saturation. Mean (±SD) total ammonia was 0.12 (± 0.06) mg/L and did not exceed 0.20 mg/L in any tank sample.

EE2 analysis

ELISA results of SPE-treated samples were not adjusted for spike recovery. Background EE2 detection in all samples from control tanks and incoming dilution water was below the the method detection limit. Mean (± SD) measured stock bottle concentration was 98% (± 23%) of nominal (3769 ± 917 ng/L). Mean (± SD) measured EE2 concentrations for the low and high exposure treatments were 66% (± 34%) and 52% (± 22%) of nominal, respectively (1.3 ± 0.67 ng/L and 5.2 ± 2.2 ng/L).

Despite lower than expected concentrations and high variability among tank samples, overall, these analyses confirm that the EE2 stock bottles were prepared correctly and that the exposure system was performing the correct dilutions, resulting in experimental treatments that can be qualitatively characterized as control, low, and high EE2 exposures. Because there was less variation among ELISA measurements of samples not treated with SPE concentration, and a lack of EE2 signal in SPE-treated control tank and LSW samples analyzed using LC/MS for a related study (personal communication with Brett Blackwell), it seems likely that variability among these

measurements may have been related to the SPE or ELISA procedures, rather than fluctuating exposure tank concentrations.

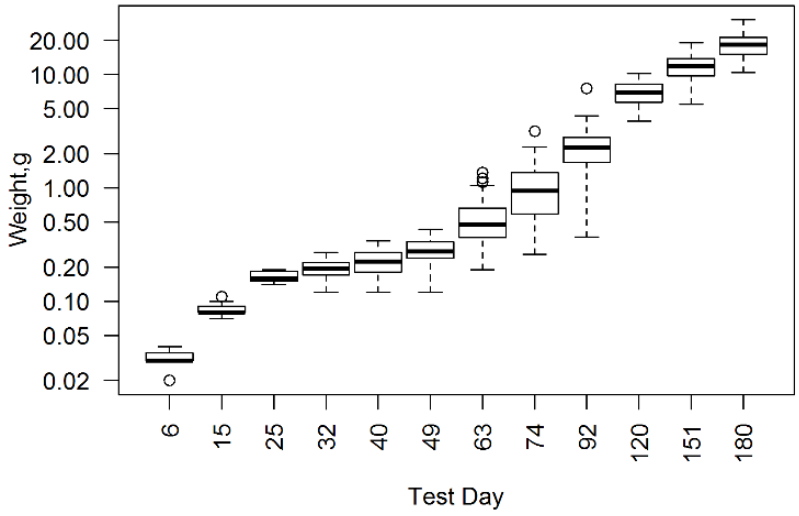
There was no significant difference in measured EE2 concentrations from samples collected just prior to the sterilization procedure compared with samples collected immediately after sterilization ($p < 0.05$), suggesting no substantial degradation of EE2 occurred in the system over time.

Growth and survival

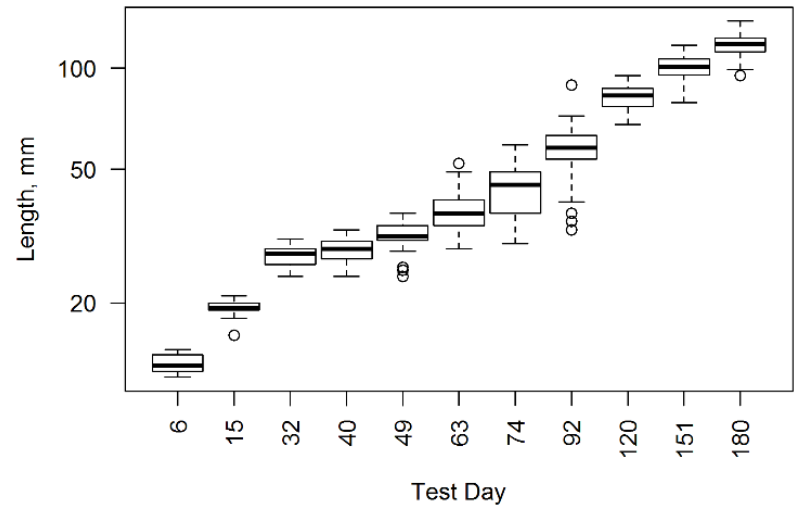
There were no significant differences in weights or lengths among treatments at most time points, with the exception of test day 120, when mean wet weight in the high EE2 exposure group (6.2 g) was significantly lower ($p = 0.02$) than the control group (7.2 g). The lower weight in the high exposure treatment may have been a result of non-specific systemic toxicity caused by the high EE2 concentration, since poor feeding behavior was observed in that group starting on day 82. The average mortality rate in the control group was 10% over the entire experiment. Between test days 28 and 34, an unknown disease caused increased mortality in two high treatment tanks (31% and 44%) and three low treatment tanks (18%, 19%, and 29%). Average mortality rate in all other treatment and control tanks between test days 28 and 34 was 3%. Poor feeding behavior was observed in some fish from each tank during the diet transition from *Artemia* to *Lumbriculus* (test days 18 through 47), which may explain a slightly decreased average growth rate measured between test days 32 and 63 (Fig. 7).

Figure 7. Box plots of weight (A) and total length (B) of smallmouth bass for 180 days.

A.



B.



Each box is a composite of data points from all treatments. The boxes represent the 25–75th percentiles; the heavy lines within the boxes are the medians; the dashed lines represent the highest values still within 1.5x interquartile range, and outliers are indicated by open dots.

Histological results

Gonads were present as elongated paired organs, each individually connected to the dorsal peritoneum by a single gonadal mesentery (Fig. 8). The two gonads were joined at a point near the urogenital pore. Each gonad extended anteriorly along the swim bladder and tapered to a narrow diameter at a point approximately mid-way between the posterior edge of the operculum and the vent (Fig. 9). The anterior-most tips of the gonads did not contain germ cells, only somatic cells and blood vessels.

Descriptions of the progression of development of undifferentiated gonads into ovaries and testes in the absence of estrogenic exposure and in the presence of EE2 are summarized in the following section. Proportions of each phenotype observed in each age group of control or EE2-exposed fish are found in Figure 10A and 10B, respectively. The stages of gonadal and germ cell development observed at each time point are summarized in Table 5.

Figure 8. Cross section through smallmouth bass collected on test day 32, showing position of undifferentiated gonads (g) in relation to the peritoneal wall (pw), swim bladder (sb), liver (lv), and stomach (s). H&E Stain

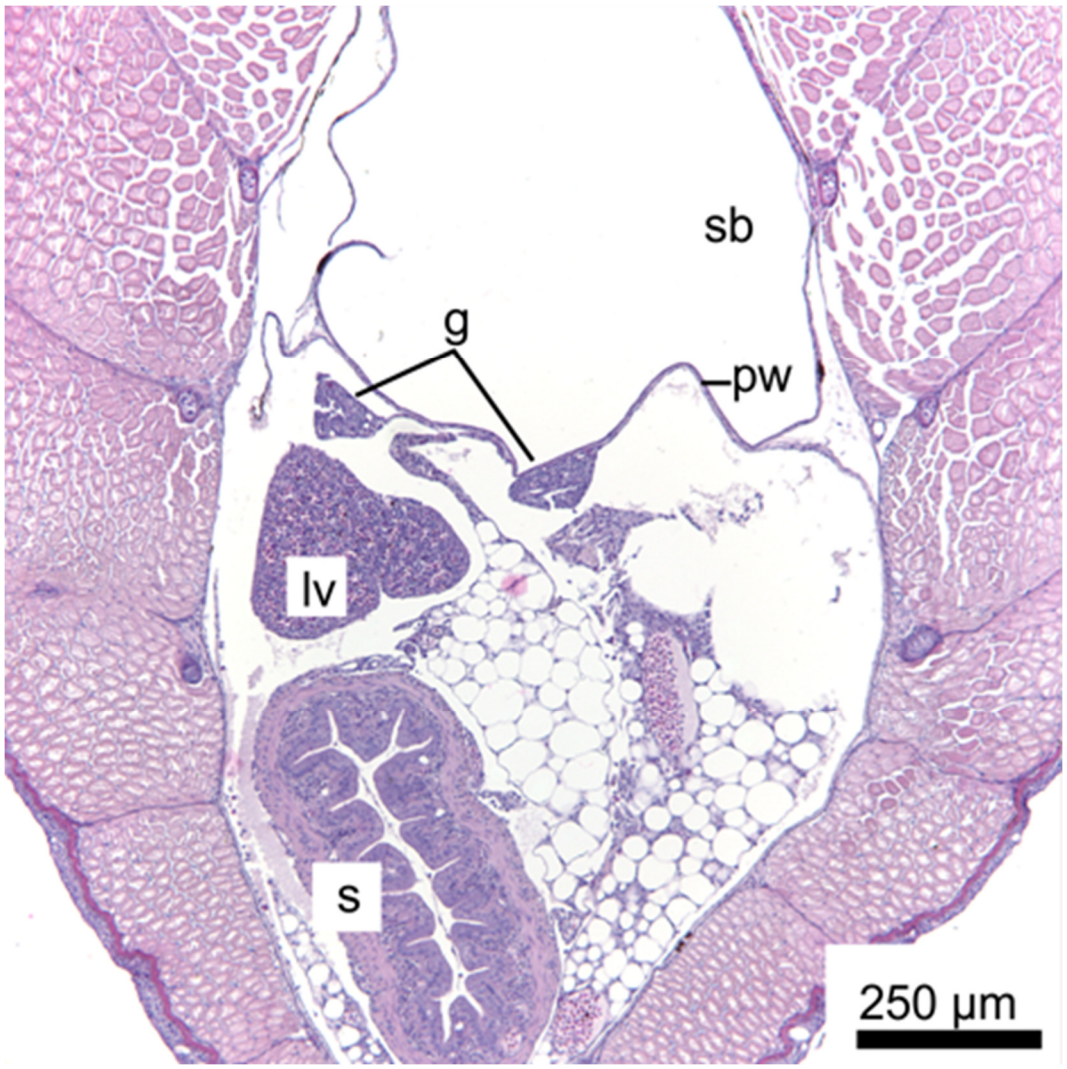
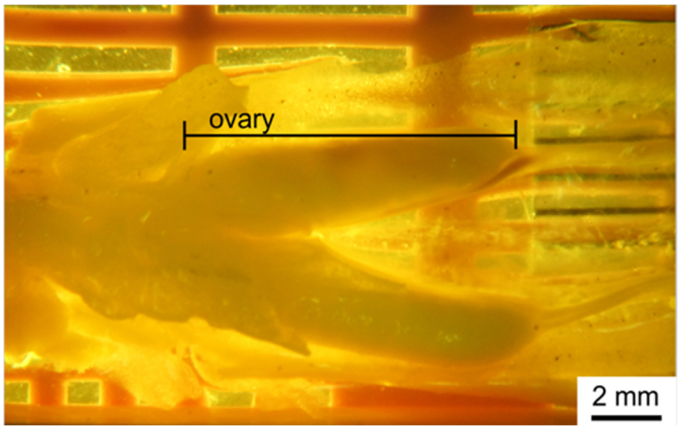


Figure 9. Gross morphology of ovaries (A) and testes (B) in control smallmouth bass collected on test day 180, after fixation in Bouin's fixative.

A



B

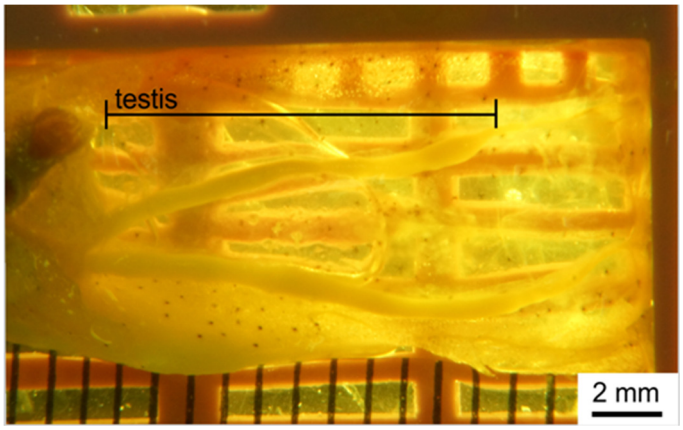
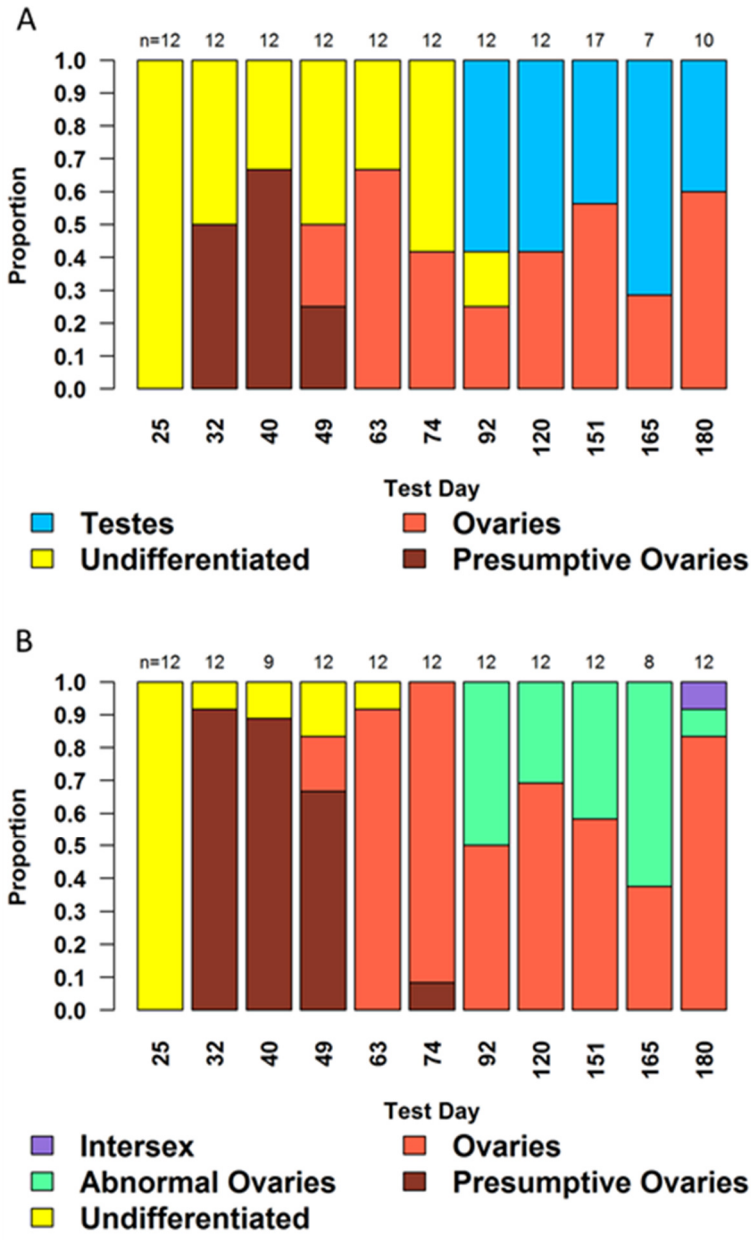


Table 5. Stages of gonadal differentiation and germ cell development in bass reared in the absence and presence of EE2.

Test day	CONTROL			EE2-EXPOSED
	Undifferentiated	Ovaries/oogenesis	Testes/spermatogenesis	
24	Small organs; few germ cells; small somatic projections (*n* = 6 of 12)	Undifferentiated characteristics	Undifferentiated characteristics	Ovarian and undifferentiated characteristics similar to control groups; no testicular characteristics
32	Slightly ↑ gonad size, blood vessel size, and germ cell *n*	Somatic projections form ovarian cavity; ↑ germ cell *n*		
40		Clusters of meiotic germ cells		
49		Few chromatin nuclear oocytes (CN)		
63		Many CN oocytes; folliculogenesis		
74		Early and late stage perinucleolar (PN) oocytes		
92	No undifferentiated gonads	Late stage PN oocytes predominant	Small spermatic tubules	Abnormal characteristics (*n* = 26 of 68): small ovary size, few oocytes, fibrosis
120		Vacuolated perinuclear stage; ↑ PN oocyte size	Pre-meiotic germ cells, early spermatogenesis	
151		↑ PN oocyte and ovary size	Multiple stages of spermatogenesis	
165			Mature spermatozoa in tubules (low number)	
180				Intersex (*n* = 1 of 12, day 180)

Figure 10. Proportions of gonadal phenotypes observed in control (A) and EE2 exposed (B) smallmouth bass.



UNDIFFERENTIATED GONADS (CONTROL FISH)

Undifferentiated gonads were defined as those with no sex-specific characteristics, and were observed in all fish collected on test day 25 (n = 12), and in 45% of fish collected on test days 32 through 74 (n = 60; Table 5, Figure 11A). Germ cells were identified in all undifferentiated gonads as 10-13 μm diameter cells with large, round to oval nuclei with regular perimeters and a single small or unapparent nucleolus. The nucleus was clear and slightly granular, and the cytoplasm was slightly basophilic, consistent with descriptions for other gonochoristic species [6, 47]. It is unknown whether germ cells observed at this stage were bipotential or differentiated into oogonia or spermatogonia; there were no identifiable sex-specific germ cell characteristics at this level of microscopy. Undifferentiated gonads in fish collected from test days 25 through 63 were small (117-215 μm diameter), with only 1-4 germ cells in each transverse section, and small blood vessels (8-13 μm). Undifferentiated gonads in fish collected on day 74 were slightly larger (118-259 μm), had larger blood vessel diameters (18-36 μm), and had more germ cells compared with earlier age groups. Six of the 12 undifferentiated gonads observed on test day 25 also had small somatic projections that may have signified the early development of the ovarian cavity, but they had no other ovarian characteristics, so they were categorized as undifferentiated.

Figure 11. Undifferentiated gonads and ovarian differentiation in smallmouth bass reared in clean water from swim-up (test day 0) through 180 days.

(**A)** Day 25, undifferentiated gonad

(B) Day 32, undifferentiated gonad somatic projections

(C) Day 40, presumptive ovary with germ cell proliferation and early meiosis

(D) Day 49, definitive ovary with early prophase oocyte

(E) Day 63, ovary with chromatin nucleolar oocytes

(F) Day 74, ovary with late perinucleolar oocytes

Abbreviations:

pw peritoneal wall

gc germ cell

bv blood vessel

sp somatic projection

sc somatic cell

oc ovarian cavity

me meiotic germ cell cluster

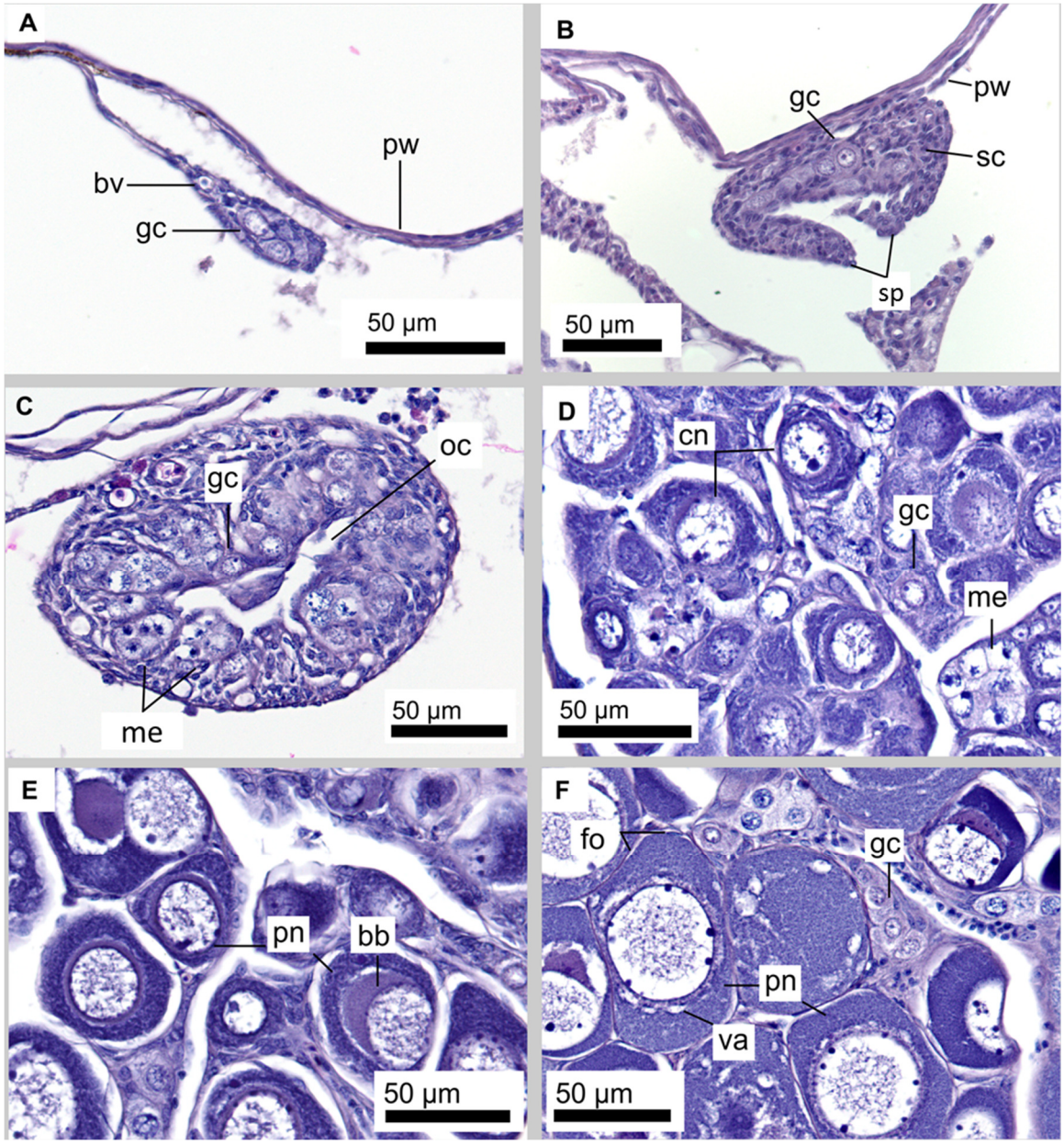
cn chromatin nucleolar oocyte

pn perinucleolar oocyte

bb Balbiani body

va vacuole

fo early follicle complex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OVARIAN DEVELOPMENT (CONTROL FISH)

Somatic projections first observed in some undifferentiated gonads on day 25 were more pronounced by day 32, and appeared to join together, forming partial or complete ovarian cavities in 6 of 12 of fish (Table 5, Fig. 11B). These gonads were considered presumptive ovaries, and also had larger cross sectional diameters (148-212 μm), larger blood vessels (23-34 μm), and slightly more germ cells in each section (4-5), compared with completely undifferentiated gonads. Presumptive ovaries were identified in 47% of fish collected on test days 32 through 49 (n = 36; 0.12 – 0.39 g wet weight; 24.0-35.0 mm total length). Cross sectional diameter increased to 125-250 μm on day 40 and 178-235 μm on day 49, concurrently with increased germ cell proliferation. Germ cell nests were clearly visible in presumptive ovaries collected on days 40 and 49, including at least one cluster of germ cells in early stages of meiotic prophase (Fig 11C), which were presumably primary oocytes, but were not yet distinguishable from early stages of meiotic spermatocytes.

The earliest identifiable oocyte stage was early diplotene (chromatin nucleolar) which was distinguished from pre-meiotic germ cells by nuclei with lampbrush formations and a single, prominent nucleolus (Fig. 11D). Ovaries with a small number of chromatin nucleolar oocytes, in addition to many meiotic germ cell nests, were observed in three of the 12 fish collected on test days 49 (0.19-0.39 g wet weight; 28.5-35.0 mm total length), and were also typically larger (178-235 μm diameter) than the undifferentiated gonads in the same age groups (71-215 μm). 47% of fish collected on test days 49 through 180 had ovaries (n = 94). Oogenesis proceeded rapidly, so that 8 of

the 12 of fish collected on test day 63 (0.29-0.88 g wet weight; 31.0-45.0 mm total length) had ovaries with increased proportions of chromatin nucleolar oocytes ranging in size from 21-50 μm, and with one to multiple nucleoli. Two fish collected on day 63 also had low numbers of perinucleolar oocytes, which were approximately the same size as the chromatin nucleolar stage oocytes, but which had multiple nucleoli around the perimeter of the nucleus (Fig. 11E). Perinucleolar oocytes were also least partially surrounded by basement membrane and granulosa cells, signaling the onset of folliculogensis (Fig. 11F). The number and size of perinucleolar oocytes (41-48 μm) increased in fish collected on test day 74 (0.29-2.09 g wet weight; 32-56 mm total length; Fig 11F), which also had larger ovaries (389-709 μm) and blood vessels (32-71 μm) compared with earlier age groups. Large numbers of late stage perinucleolar oocytes of increased diameter (62-88 μm) with Balbiani bodies appearing as dark crescents adjacent to the nuclear envelope were present in fish collected on day 92 (0.48-3.91 g wet weight; 45-65 mm total length; 483-1065 μm ovarian diameter; Fig. 11E). Perinucleolar stage oocytes continued to increase in size (100-116 μm), and appeared vacuolated (Fig. 11F) in fish collected on day 120 (4.03-10.00 g wet weight; 69-93 mm total length), which also had larger ovaries (824-1568 μm diameter) than the previous age group. Primary oocytes did not advance beyond the vacuolated perinucleolar stage, but increased slightly in size to a maximum range of 121-143 μm on day 151. Ovarian diameter also continued to increase from day 151 (1526-2022 μm) to day 180 (1742-2451 μm). In all ovaries dominated by late-stage primary oocytes, small numbers of germ cells and early stage oocytes, both singular and within nests, were also present (Fig. 11F). Between test days

49 through 180, the percent of control fish that developed ovaries was not significantly different than 50%, although 95% confidence intervals were wide (Table 6).

Table 6. Percentages of control and EE2-exposed fish (low exposure treatment only; 2 ng/L nominal) that developed normal ovaries during experiment 1, and 95% confidence intervals of the percentages.

Test Day	Treatment	*N*	Percent of fish with normal ovaries	95% confidence interval	
				Lower CI	Upper CI
92	Control	12	25%	7%	57%
	EE2	12	50%	25%	75%
120	Control	12	42%	17%	71%
	EE2	12	67%	35%	89%
151	Control	17	53%	29%	76%
	EE2	12	58%	29%	84%
165	Control	7	29%	5%	70%
	EE2	8	38%	10%	74%
180	Control	10	60%	25%	75%
	EE2	12	83%	35%	89%

Age groups shown are those in which both definitive ovarian and testicular differentiation was observed in control groups. Percentages of fish that developed normal ovaries were not significantly different than 0.5 for any age group ($p > 0.05$).

TESTICULAR DEVELOPMENT (CONTROL FISH)

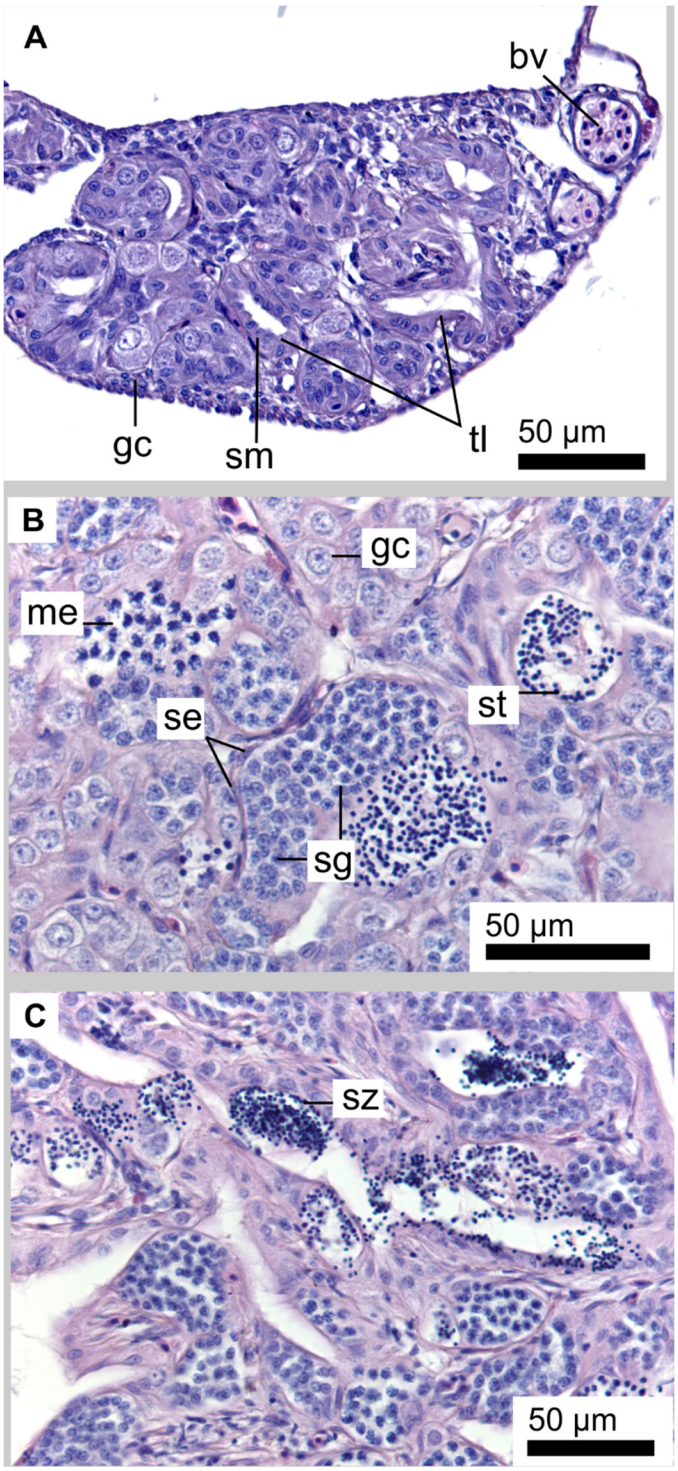
Testicular differentiation was first identified in fish collected on day 92 (0.48-3.91 g wet weight; 45-65 mm total length), based on the presence of small spermatic tubules in parts or all of the gonadal tissue (Table 5, Fig. 12A). Tubules consisted of cuboidal somatic cells arranged in a single layer with a basement lamina on the external perimeter and a small interior lumen. A low number of germ cells, presumably spermatogonia, were also present in the tubules (Fig. 12A). Somatic cells were distinguishable from germ cells by their smaller size (7-8 μm dia.) and smaller, oval, basophilic nucleus (Fig. 12A), although it unknown whether they were presumptive or differentiated Sertoli cells at this stage. At the onset of spermatogenesis, testes increased in size rapidly, with maximum observed diameter ranging from 170 to 325 μm on test day 92, 309-495 μm on day 120, 481-1405 μm on day 151, and 936-1159 μm on day 180. Proliferation of pre-meiotic germ cells was apparent in fish collected on day 120 (4.03-10.00 g wet weight; 69-93 mm total length), concurrent with increased size of spermatic tubules. The onset of spermatogenesis was also first observed in a single fish collected on day 120, identifiable by several cysts containing meiotic germ cells in the center region of the gonad (Fig. 12B). Five out of 6 male fish collected on test day 151 (7.18-19.04 g wet weight; 84-117 mm total length), had testes with spermatocysts in multiple stages of spermatogenesis, including spermatogonia A, spermatogonia B, zygotene meiosis, diplotene spermatocytes, and small numbers of spermatids (Fig. 12B). Spermatocysts were surrounded by Sertoli cells and a basement lamina, with small blood vessels interspersed between cysts (Fig. 12B). Leydig cells were not clearly defined. Spermatocyst

maturation appeared to progress from the center of the testis outward: the most advanced stages of spermatogenesis were present in the center region of each cross-section, while only pre-meiotic germ cells were present around the perimeter. The germinal epithelia of testes observed in fish collected on test days 165 (10.25-18.14 g wet weight; 97-110 mm total length) and 180 (13.26-30.30 g wet weight; 105-138 mm total length) were dominated by germ cells and meiotic cysts, and small amounts of mature spermatozoa (Fig. 12C) were observed in center-most tubules of five of nine male fish.

Figure 12. Testicular differentiation in smallmouth bass reared in clean water from swim-up (test day 0) through 180 days.
(A) Day 92, testis with early spermatic tubules
(B) Day 120, spermatocysts enclosing clusters of spermatogonia types A and B, meiotic spermatocytes, and spermatids
(C) Day 180, testis with mature spermatozoa.

Abbreviations:

gc germ cell
bv blood vessel
sm somatic cell
tl tubule lumen
sg spermatogonia types A and B
me meiotic spermatocytes
se Sertoli cells
st spermatids
sz spermatozoa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GONADAL DEVELOPMENT IN EE2-EXPOSED FISH

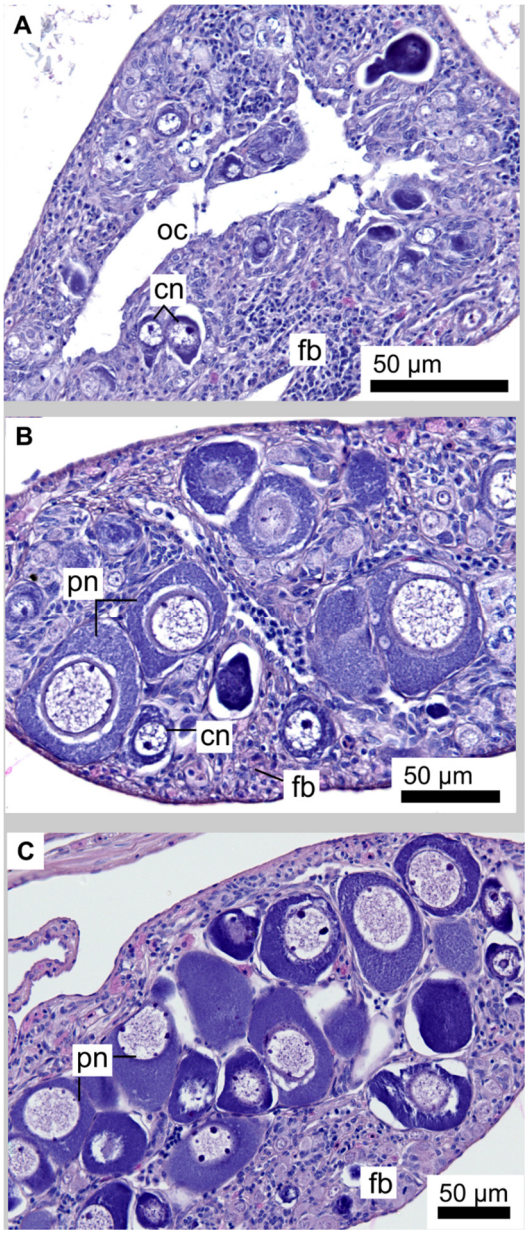
An initial evaluation of gonadal tissues from both low (n = 8) and high (n = 3) EE2 exposure treatments collected on day 165 revealed that all fish exposed to EE2 had developed ovaries, including, presumably, the genetic males. Because complete sex reversal represents the most extreme possible histologic endpoint under the present experimental design, only the tissues from the low EE2 exposure groups were evaluated and reported here.

Gonadal development in EE2-exposed fish was initially indistinguishable from the control groups; all fish collected on day 25 (0.16-0.19 g wet weight; 25.0-26.0 mm total length) had small, undifferentiated gonads, with subtle indications of somatic projections also apparent in some gonads. Undifferentiated gonads were also observed in at least one fish collected on days 32, 40, 49, and 63, but the phenotypic sex ratio was strongly female-biased in each age group (83-92%). Presumptive ovaries were present in fish collected on days 32 and 40 (0.16-0.33 g wet weight; 25.0-33.0 mm total length, 86-299 μm ovarian diameter), characterized by developing or completely enclosed ovarian cavities, germ cell proliferation, and occasionally, one or more clusters of meiotic germ cells in stages too early to identify as oocytes. Definitive ovaries were first identifiable in EE2-exposed fish collected on day 49 (0.12-0.43 g wet weight; 24.0-37.0 mm total length), characterized by the presence of a small number of early chromatin nucleolar oocytes, but there was no increase in diameter (135-257 μm) compared with the previous age group. Ovaries continued to develop similarly to control fish between test days 63 and 74 (0.24-1.79 g wet weight; 30.0-56.0 mm total length; 164-566 μm maximum

ovarian diameter). Among EE2-exposed fish collected between test days 92 and 180, 98% of fish developed ovaries. During this time period, 61% of the ovaries were similar to those in the control groups, and 38% (n = 68) had abnormal characteristics. Abnormal ovaries had fully developed ovarian cavities, but had smaller cross sectional diameters (373-1781 μm) compared with normal ovaries in the same age groups (624-2298 μm), increased fibrotic cells around the perimeter, and very few oocytes, found mainly in the center region (Fig. 13A-C). Notably, the proportion of fish that developed normal ovaries (61%) was not significantly different than 50%, which would support the notion that the fish with abnormal ovaries were sex-reversed males (Table 6).

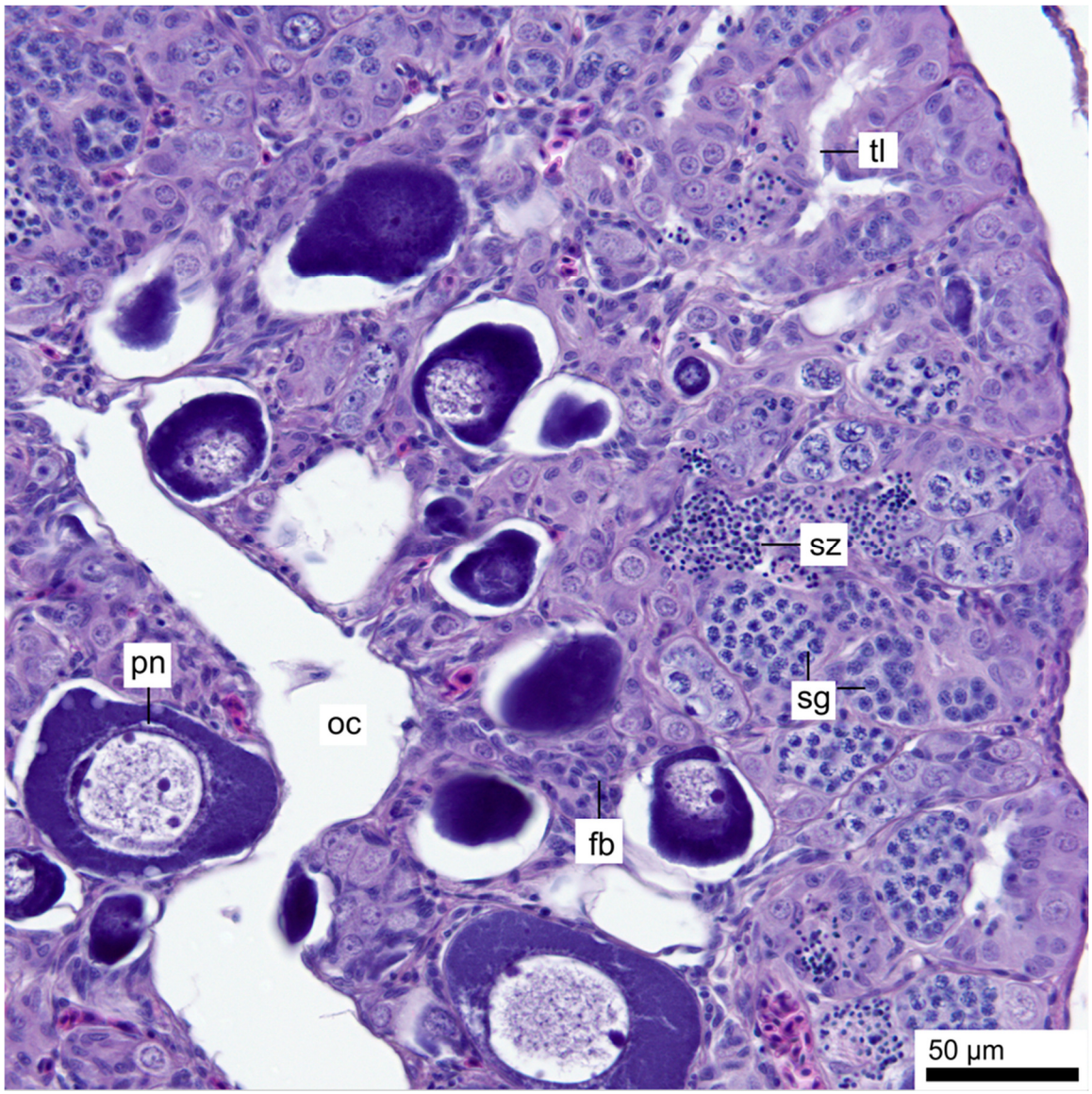
On day 180, a single individual had intersex gonads, with center regions that contained ovarian tissue with several perinucleolar oocytes and fully developed ovarian cavities, and, around the perimeter, testicular tissue that included spermatic tubules with spermatocysts in all stages of development up to and including small numbers of spermatids, as well as some fibrosis (Fig. 14). The pattern of oocytes in the center and fibrotic tissue around the perimeter of this intersex gonad was notably similar to the abnormal ovary phenotype, although no other abnormal ovary had testicular characteristics.

Figure 13. Development of abnormal ovaries in smallmouth bass exposed to EE2 (2 ng/L nominal). Abnormal ovaries were observed in 38% of EE2-exposed fish collected on test days 92 **(A)**, 120 **(B)**, 151 (not shown), 165 (not shown), and 180 **(C)**.



Abbreviations: **oc**, ovarian cavity; **cn**, chromatin nucleolar oocytes; **fb**, fibrotic tissue; **pn**, perinucleolar oocytes

Figure 14. Intersex gonad observed in a single smallmouth bass exposed to EE2 (2 ng/L nominal).



Abbreviations: **oc**, ovarian cavity; **pn**, perinucleolar oocyte; **sg**, spermatogonia type A and B; **tl**, spermatic tubule lumen; **sz**, spermatozoa

Conclusions

To our knowledge, this is the first study to describe early life stage smallmouth bass gonadal differentiation and germ cell development under the absence and presence of exogenous estrogen. Results from control groups include histological images of normal gonadal tissue in stages of development ranging from undifferentiated to just prior to maturity. The patterns of development we observed were generally similar to those described for other model species [6, 52, 70, 71], especially those for the closely related bluegill sunfish [72]. Early signs of presumptive ovarian differentiation were observed at test day 32, and ovarian characteristics were unambiguous by day 49. Ovarian cavity development was observed at approximately the same time as the onset of germ cell proliferation. The ovarian cavity formed when extensions of somatic cells on each side of the presumptive ovary joined to create an enclosed cavity. This pattern of ovarian cavity development also occurs in bluegill sunfish (*Lepomis macrochirus*, [72]) and pejerrey (*Odontesthes regia*, [51]), but is different than in other species (e.g. fathead minnow and medaka), in which the edges of the gonad fuse directly to the peritoneal wall to form a cavity between the ovary and the peritoneum [41, 70].

Testicular differentiation was not apparent until test day 92. It has been noted in many other species that ovarian differentiation also occurs some weeks or months prior to the onset of testicular differentiation [6, 52]. The lack of testicular characteristics in undifferentiated gonads of presumed males between test days 32 through 74 indicates that testes cannot be phenotypically identified in smallmouth bass prior to the onset of spermatic tubule formation and germ cell differentiation, unlike males of some other

species, which can be identified in early stages by the organization of the germ cells relative to somatic cells, or the number of attachment points to the peritoneal wall [42, 70]. This finding may have relevance for future studies on smallmouth bass, in that female-biased phenotypic sex ratios will not be detectable in age groups with only undifferentiated gonads.

The documentation of an early developmental stage in which all gonads were undifferentiated, followed by direct development into either ovaries or testes, is consistent with the differentiated gonochorism type of developmental ontogeny, like that of closely related species (i.e. largemouth bass and bluegill sunfish; [46, 72]). This suggests that, in smallmouth bass, TOs are unlikely to arise as a consequence of ovarian-to-testicular transition, as has been observed in zebrafish.

Our results indicated that from test days 49 through 74 (the time points at which definitive ovaries, but not testes, were identifiable in control groups), 92% of fish in the low EE2 exposure treatment had ovaries that were histologically indistinguishable from those in control fish, and that from days 92 through 180 (time points at which testicular differentiation was apparent in control groups), 98% of fish in the low EE2 exposure treatment had ovaries. Based on ELISA results discussed previously, this suggests that 90 days of early life exposure to EE2 in the range of 0.5 to 2.7 ng/L EE2 (1.3 ng/L mean measurement) caused presumptive sex reversal in genetic males, which is within the range of sensitivities determined for other species [7, 8, 12].

Abnormal phenotypes (small cross sectional area, decreased number of oocytes, and increased fibrosis) were observed in 38% of ovaries in the EE2 exposure groups

between days 92 and 180 (n = 68). In comparison, testes developed in 41% of control fish between test days 92 and 180. We speculate that abnormal ovaries may have developed in sex-reversed genetic males and normal ovaries developed in genetic females, although genetic sex was not known. Although the observation of abnormal ovaries on day 92 corresponded with the end of the EE2 exposure (day 90), the narrow window between these events suggests their relationship was coincidental, rather than causative. The first observation of abnormal ovaries also corresponded with the first observation of definitive testicular differentiation in control groups (test day 92), which suggests that, in EE2-exposed males, the cellular pathways involved in testicular development may not have been completely inhibited. The finding of ovaries in presumably sex-reversed males that can be histologically distinguished from ovaries in presumed genetic females has not been previously described in other species, to our knowledge, although it was noted that within a group of EE2-exposed sex-reversed roach, the stages of ovarian development varied more widely than in the control group [42].

Fully intersex gonads, with regions of well differentiated ovarian and testicular tissue, were observed in only one fish, collected on day 180. In their central regions, these gonads resembled the abnormal ovary phenotype, with testicular regions around the perimeter. This observation is notable because it was the only EE2-exposed fish that developed gonads with any testicular characteristics, although having only occurred in only one fish, the intersex phenotype cannot be definitively attributed to EE2 exposure.

TOs were not observed in any EE2-exposed fish in developmental stages where TOs would presumably be detectable (i.e. after testicular differentiation, day 92), and

therefore does not provide direct evidence to link TOs in wild adult bass with exposure to estrogenic compounds. However, the basic knowledge about smallmouth bass gonadal differentiation, germ cell development, and the effects of early life exposure to EE2, is information that will be useful for more effectively interpreting potential cases of endocrine disruption in wild populations, and for making comparisons between smallmouth bass and other more extensively studied models for endocrine disruption.

IV. Effects of environmentally relevant concentrations of 17α-ethinylestradiol on smallmouth bass gonadal development

The goal of this study was to evaluate the effects of early life exposure to EE2 on smallmouth bass gonadal development at concentrations lower than those that caused sex reversal in Chapter III. A range of concentrations was chosen with the expectation of collecting data from at 1) least one treatment group with no significant differences in gonadal development compared with control groups (a no-observed effect concentration; NOEC), 2) a high-concentration treatment group in which all fish developed ovaries (i.e. 100% sex reversal, to provide direct comparison with the lab study discussed in Chapter III), and 3) at least one treatment group with intermediate effects (a lowest-observed effect concentration; LOEC).

The potential intermediate effects that were expected included a concentration-dependent increase in sex reversal, or a concentration-dependent increase in the degree of gonadal feminization. It was expected that, if observed, partially feminized testes might appear similar to the testes with testicular oocytes (TOs) observed in wild-collected adult smallmouth bass, and therefore provide evidence that early life exposure to EE2 can cause TOs in wild populations. The lowest test concentrations (0.1, and 0.4 ng/L EE2, nominal), were in the range of measurements of EE2 that have been detected near wastewater treatment effluent outflows in North America and Europe.

It was calculated that evaluating the phenotypic sex of 16 fish from each of 3 replicate tanks per treatment would provide the ability to detect sex reversal of 50% or

more of the males with statistical power of at least 80%. Growth rate estimations from previous work (Chapter III) were used to predict the maximum number of fish per tank at each time point that would not exceed the target biomass loading rates. Therefore, the timing of sample collections for this test was designed to maximize sample sizes available on test day 90 for statistical analysis of proportions of gonadal phenotypes, and to grow out a small number of fish until test day 120 for a qualitative description of gonadal phenotypes. This experiment was carried out at the US EPA Mid-Continent Ecology Division, Duluth, MN.

Methods

Collection of wild smallmouth bass

Wild smallmouth bass yolk-sac fry were collected from two nests in Pike Lake (9 mi W of Grand Marais, Cook County, MN) and transported to the laboratory on test day 0, as previously described (Chapter III). Fry from both nests appeared to be within 7 days post-hatch, although those collected from nest #1 were slightly smaller, had larger yolk sacs, and were less mobile compared with those collected from nest #2. Survival during transportation was excellent (> 99%).

Experimental design and exposure conditions

EE2 stock bottles (1080 ng/L nominal concentration) were prepared every 14 days, and a flow-through exposure system was calibrated to dilute EE2 stock to nominal concentrations of 0.1, 0.4, 1.1, and 3.3 ng/L EE2 as described previously (Chapter III).

Correct dilution ratios were confirmed volumetrically both at the beginning of, and midway through the experiment.

Triplicate exposure chambers were set up for each treatment group, and consisted of preassembled 2.5-gallon glass aquaria with silicone-sealed joints, clear acrylic covers, and stainless steel outflow standpipes positioned to allow a filled volume of 7 L. Test organisms were added to exposure chambers in a randomized block design. Fry from nest #1 were placed in one of the three replicate chambers of each treatment, and fry from nest #2 were placed in the remaining two replicate chambers of each treatment, distributed in groups of 10 until each tank contained 100 fry. Test organisms were continuously exposed to EE2 or clean water starting on test day 0 for 100 days, and then reared for an additional 20 days in clean water.

In order to meet the target tank temperature of 22 °C, incoming water was tempered to 23.0 ° C using electronically controlled mixing valves with an alarm threshold of ±1° C. The flow-through rate was initially set at 12 volume additions per 24 hours (60 mL/min) and increased to 80 mL/min on test day 50, to reduce loading below 0.5 g per 1 L flowing through in 24 hours, as recommended by ASTM guidelines for early life-stage tests [69]. Tanks were supplied with filtered air through disposable borosilicate glass Pasteur pipettes. Temperature and dissolved oxygen were measured daily in at least one tank per treatment; means ± SD were 21.9 ± 0.3° C and 7.1 ±0.7 mg/L, respectively. pH was measured weekly in at least 1 tank per treatment and averaged 7.1 ± 0.2. Total ammonia was measured in each tank at three time points during

the experiment, and averaged 0.24 ± 0.07. The light cycle was maintained at 16:8 light:dark throughout the experiment.

Fish from nest cohort #2 swam up and began to feed exogenously on test day 1, and fish from nest cohort #1 swam up on test day 3. From swim-up through test day 51, fish were fed newly hatched brine shrimp nauplii (*Artemia spp.*) at a target ration of 20% average wet-weight, divided into two or three feedings per day. Starting on day 52, 1 g blackworms (*Lumbriculus spp*., California Blackworm Co., Fresno, CA), were provided to each tank, and the remainder of the 20% daily ration was made up with brine shrimp nauplii. The proportion of the daily ration that consisted of blackworms was gradually increased until test day 67, when fish in all tanks were able to consume the total daily ration in a single feeding of blackworms. Beginning on test day 67, the daily ration was readjusted weekly in order to maintain an overall average specific growth rate that would not exceed target biomass loading rates, while maximizing sample numbers for histological evaluation. The actual daily ration was 20% on test day 67, and was decreased gradually to 13% by test day 120.

Average fish weight was estimated on test day 0 by the average blotted wet weight of three replicates of 5 fish from each nest. Thereafter, the average total biomass of each tank was estimated weekly by measuring the blotted wet weight of euthanized fish collected for histological evaluation, or the live weight of a subsample of arbitrarily selected fish from each exposure chamber. Average individual live weights were estimated from the composite weight of 3-7 fish from at least one tank per treatment, by netting them into a tared beaker containing clean Lake Superior water, minimizing the

amount of water transferred into the beaker. Based on previous work (Chapter III), it was assumed that the maximum nominal concentration of 3.3 ng/L EE2 would not affect growth rates, so average weights from all chambers were pooled, and identical feeding rates were provided to each chamber. As tank biomass increased, fish were removed to reduce loading below 5 g of biomass per 1 L of standing volume, per ASTM guidelines [69].

Tanks were cleaned daily by scrubbing and siphoning debris and uneaten food. A sterilization procedure was performed every two weeks during the exposure period to reduce biofilm growth, as described previously (Chapter III).

EE2 analysis

Using the available analytical methods, EE2 concentrations in the nominally 0.1 and 0.4 ng/L exposure tanks were not quantifiable. Therefore, only samples from the 1.1 and 3.3 ng/L (nominal) exposure tanks were analyzed for the duration of the experiment. During the EE2 exposure period, tank water samples were collected from at least one control tank, and at least one tank from the 1.1 or 3.3 ng/L (nominal) exposure treatments, plus one duplicate sample, 11 times (roughly every 1-2 weeks). Samples (50 mL for 3.3 ng/L EE2 nominal concentrations, and 150 mL for all other samples) were placed in solvent-cleaned bottles and either processed immediately or held for no more than 24 hours at 4 °C. Debris was not observed in the samples, and so filtration was determined to be unnecessary. Tank samples and standards were concentrated with solid phase extraction cartridges (Strata X 60 mg, Phenomenex Inc.) using standard procedures

including methanol elution, evaporation under nitrogen, and reconstitution in 25% HPLC-grade methanol. Each time the EE2 stock bottle was refilled, an EE2 stock sample was collected, spiked with HPLC-grade methanol to a final concentration of 25%, and analyzed without further dilution or concentration. All samples, blanks, and standards were spiked with 1.1 ng of EE2 17α-ethinylestradiol-2,4,16,16-D4 (EE2-D4, CDN Isotopes, Quebec, Canada) as an internal standard. Samples were analyzed and quantified by liquid chromatography / tandem mass spectroscopy (LC/MS/MS) using an Agilent 6410 system with atmospheric pressure photoionization (APPI) in positive ionization mode. EE2 was separated by gradient elution of methanol and water at 0.3 mL/min on a Phenomenex PFP (2.1 x 100 mm, 2.6 µm) column. Under these conditions, EE2 had a retention time of 12.0 min. Toluene was used as a dopant for APPI and was infused post column into the ion source at 0.018 mL/min. A loss of water was observed from EE2, so the [M-H20]+H ion was used as the precursor ion. Two ion transitions were monitored for both EE2 and EE2-D4 (Table 7). Measured EE2 concentrations for each exposure treatment were reported as the mean and standard deviation of all samples analyzed.

Table 7. Monitored ion transitions (m/z) for ethinylestradiol quantification

Compound	Precursor Ion (m/z)	Product Ion(s) (m/z)[a]
EE2	279.2	**133.0**, 159.2
EE2-D4	283.3	**155.1**, 161.0

[a]Quantifier ion in bold

Histological evaluation

On test days 15, 21, 30, 59, 80, 90, and 120, subsamples of 4 to 20 fish were removed from each tank and euthanized with an overdose of MS-222 (3 g/L, Western Chemical, Ferndale, WA, buffered to neutral pH with $NaCO_3$). Total lengths (to the nearest 1 mm) and blotted wet weights (to the nearest 1 mg) were collected for each fish. Heads and tails were removed, abdomens were slit ventrally, and abdominal cavities and gill cavities and were perfused with a solution of 4% paraformaldehyde in phosphate buffered saline (Sigma-Aldrich). After a 72 hour fixation period, samples were transferred to 70% ethanol for storage until processing. Gonad tissues collected on days 90 and 120 were processed, embedded in paraffin, and sectioned according to standard histological techniques described previously (Chapter III). Three fully-faced transverse sections through both gonads were evaluated for each fish. Maximum observed cross sectional diameters and oocyte diameters were measured to the nearest µm with a light microscope (Zeiss Axiovert 35) calibrated with a stage micrometer using a Spot scanner (Diagnostic Instruments) with Spot software. Gonadal phenotypes were evaluated for each fish, defined by previous work (Chapter III) on smallmouth bass reared in the absence and presence of EE2, as either undifferentiated gonad, ovary, testis, or abnormal ovary.

Statistical analysis

Analytical chemistry data were processed using MassHunter Quantitative Analysis version B.06.00 (Agilent Technologies). EE2 was quantified using an internal

standard method with EE2-D4 as internal standard. A 1/x weighting was applied to linear calibration curves to increase fit at lower concentrations.

Statistical analyses of biological data were performed with R version 3.2.2 [57]. Differences between groups were considered to be significant at an alpha level of 0.05. Blotted wet weights of fish collected for histological analysis were log-transformed to meet the assumption of normality. Dunnett's multiple comparison test was used to determine whether mean blotted weight differed between the treatment and control groups in each age group. Chi-square analysis was used to detect significant deviations from the expected proportion of fish that developed ovaries (0.5) in tissues collected on test days 90 and 120.

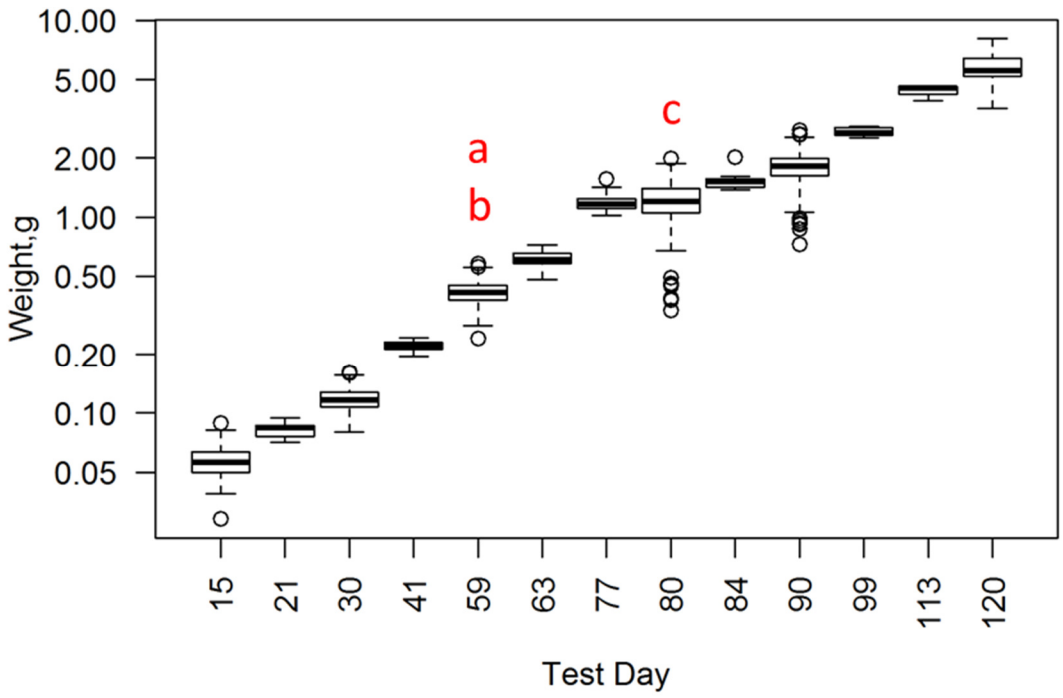
Results

Growth and survival

The average survival rate was 95% (range: 90-99%) among all exposure chambers, accounting for mortalities of unknown cause, accidental mortalities, and fish that were removed because they appeared unhealthy. The majority of all mortalities occurred during the first 30 days of the experiment. Average weights in EE2 exposure treatments were not statistically different than control groups at most time points (Fig. 15); the few exceptions were not suggestive of an EE2-related effect. On test day 59, the mean weight of the 0.1 ng/L EE2 exposure group was significantly greater ($p = 0.01$) than the control group (Fig. 15, subscript a), and the mean weight of the 1.1 ng/L group was significantly lower ($p = 0.01$) than the control group (Fig. 15, subscript b). On test

day 80, blotted weight of the 0.4 ng/L group was significantly greater (p = 0.01) than the control group (Fig. 15, subscript c).

Figure 15. Box plots of wet weights of smallmouth bass from test days 15-120.



Each box is a composite of data points from all treatments, including both control fish and fish exposed to 0.1, 0.4, 1.1, or 3.3 ng/L EE2 (nominal). Lowercase letters indicate times at which treatment groups were significantly different than the control groups ($p < 0.05$) as described in the text. The boxes represent the 25–75th percentiles; the heavy lines within the boxes are the medians; the dashed lines represent the highest values still within 1.5x interquartile range, and outliers are indicated by open dots.

EE2 analysis

EE2 was not detected in method blanks or control tank samples. Duplicate samples (n = 13) demonstrated acceptable precision with a relative percent difference (RPD) of 13.8. EE2 matrix spikes were elevated throughout the experiment, with means (SD) of 1.84 (0.30) and 5.45 (0.78) ng/L for the nominally 1.0 (n = 7) and 3.0 (n = 7) ng/L spikes, respectively. Reported tank concentrations were not corrected for apparent increased recovery of matrix spikes. The measured EE2 concentrations ranged from 1.2 to 2.0 ng/L in the nominally 1.1 ng/L exposure tanks, and from 2.6 to 5.5 ng/L in the nominally 3.3 ng/L exposure tanks, with means (SD) of 1.6 (0.2) and 4.3 (0.8) ng/L, respectively, corresponding to 143% and 130% of nominal, respectively. The average (SD) measured concentration of EE2 stock solutions was 1087 (66) ng/L (108% of nominal). Based on this average measured EE2 stock concentration and the dilution ratios of the pumps, the actual concentrations of the 0.1 and 0.4 ng/L EE2 (nominal) exposure tanks were also expected to be slightly higher than nominal concentrations.

Histological results

Ovarian differentiation was observed in 44% and 55% of control fish collected on days 90 (n = 18) and 120 (n = 11), respectively. These percentages were not significantly different than 50%, although confidence intervals were wide (Table 8).

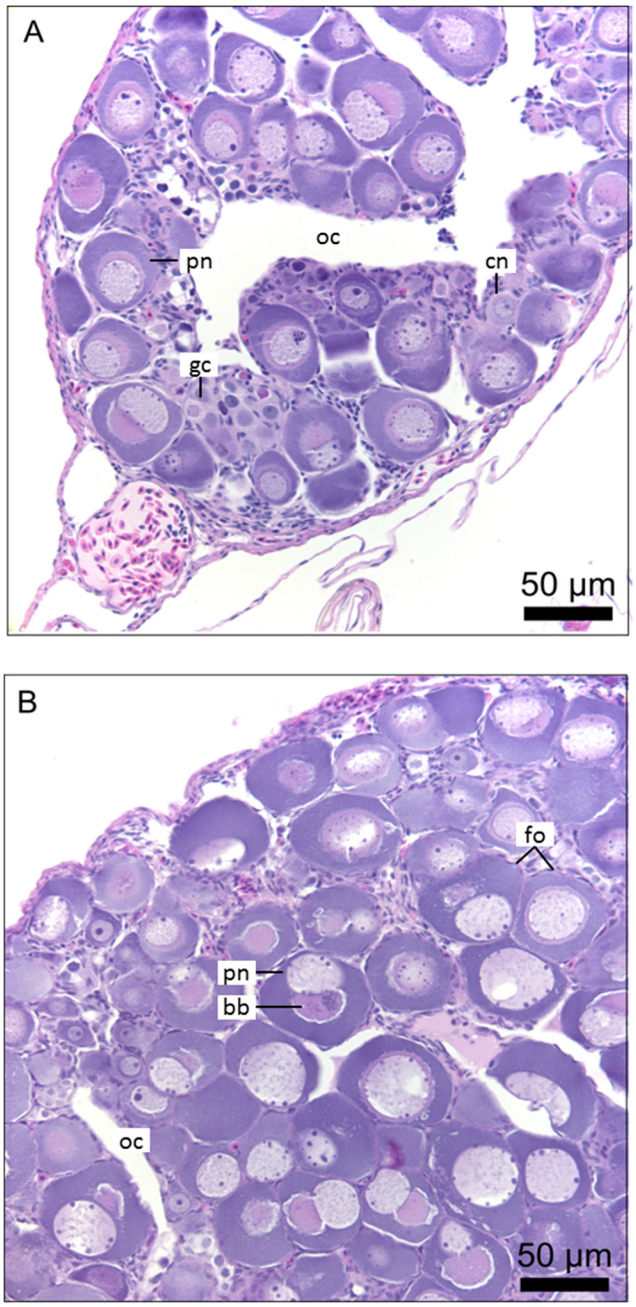
Table 8. Percentages of control and EE2-exposed fish (nominal concentrations) that developed normal ovaries during experiment 2, and 95% confidence intervals of the percentages.

Treatment	Test Day	*N*	Percent of fish with normal ovaries	95% confidence interval	
				lower CI	upper CI
Control	90	18	44%	22%	69%
	120	11	55%	25%	82%
0.1 ng/L	90	20	40%	20%	64%
	120	11	55%	25%	82%
0.4 ng/L	90	18	72%	46%	89%
	120	10	60%	27%	86%
1.1 ng/L	90	19	37%	17%	61%
	120	10	60%	27%	86%
3.3 ng/L	90	20	60%	36%	80%
	120	12	67%	35%	89%

The percent of fish that developed normal ovaries was not significantly different than 50% for any age group ($p > 0.05$).

In general, ovaries increased in size from 426-756 µm maximum cross-sectional diameter among fish collected on day 90 to 708-1215 µm among fish collected on day 120. All ovaries had fully developed ovarian cavities and were densely packed with primary oocytes in perinucleolar stage, ranging from 48-62 µm maximum observed diameter among fish collected on day 90 (Fig. 16A), and generally increasing among fish collected on day 120 (74-84 µm; Fig. 16B). Individual oocytes appeared to be at least partially surrounded by early follicle complexes, including presumptive granulosa cells and basement membranes (Fig. 16B).

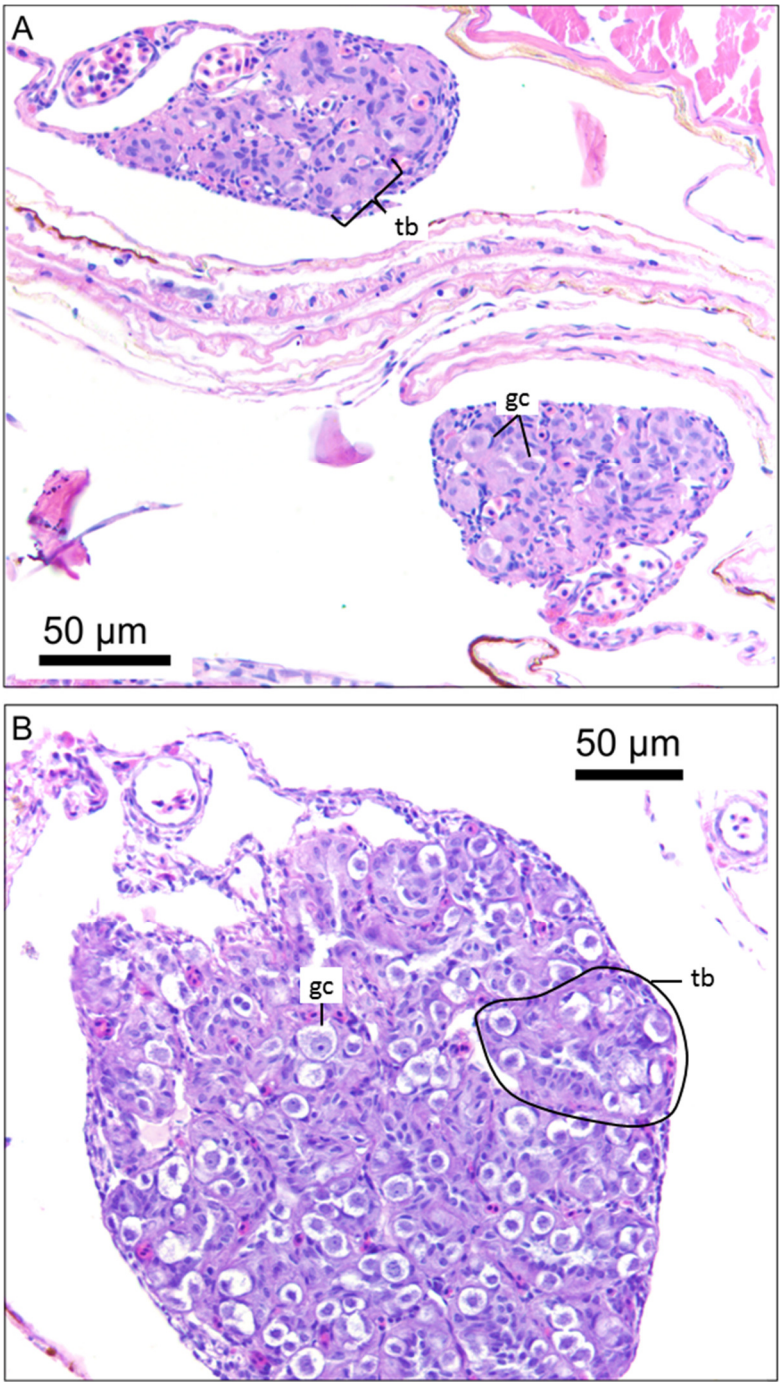
Figure 16. Ovaries in smallmouth bass reared in clean water for (A) 90 and (B) 120 days.



Abbreviations: **gc**, germ cell; **oc**, ovarian cavity; **cn**, chromatin nucleolar oocyte; **pn**, perinuclolar oocyte; **bb**, Balbiani body; **fo**, presumptive follicle complex

Among control fish collected on test day 90, all fish that did not have ovaries had undifferentiated gonads ranging in maximum diameter from 132-204 µm (Fig 17A). Early signs of presumptive testicular differentiation were present in undifferentiated gonads, but were too subtle and inconsistent to definitively characterize them as testes. Presumptive indications of testicular differentiation included early germ cell proliferation (6-24 germ cells per section) and a few, small regions of somatic cells that appeared to be organized into spermatic tubules; these cells were arranged in small circles with basement membrane around the outer edges, but a lumen was not yet present in the center. On test day 120, all gonads that were not identified as ovaries were clearly undergoing testicular differentiation, based on the presence of small spermatic tubules (Fig. 17B). Testes also had increased maximum diameters (295-417 µm) and more germ cells compared with undifferentiated gonads observed on test day 90, but spermatogenesis had not yet started.

Figure 17. Presumptive (A) and definitive (B) testes observed in control smallmouth bass collected on test days 90 and 120, respectively.

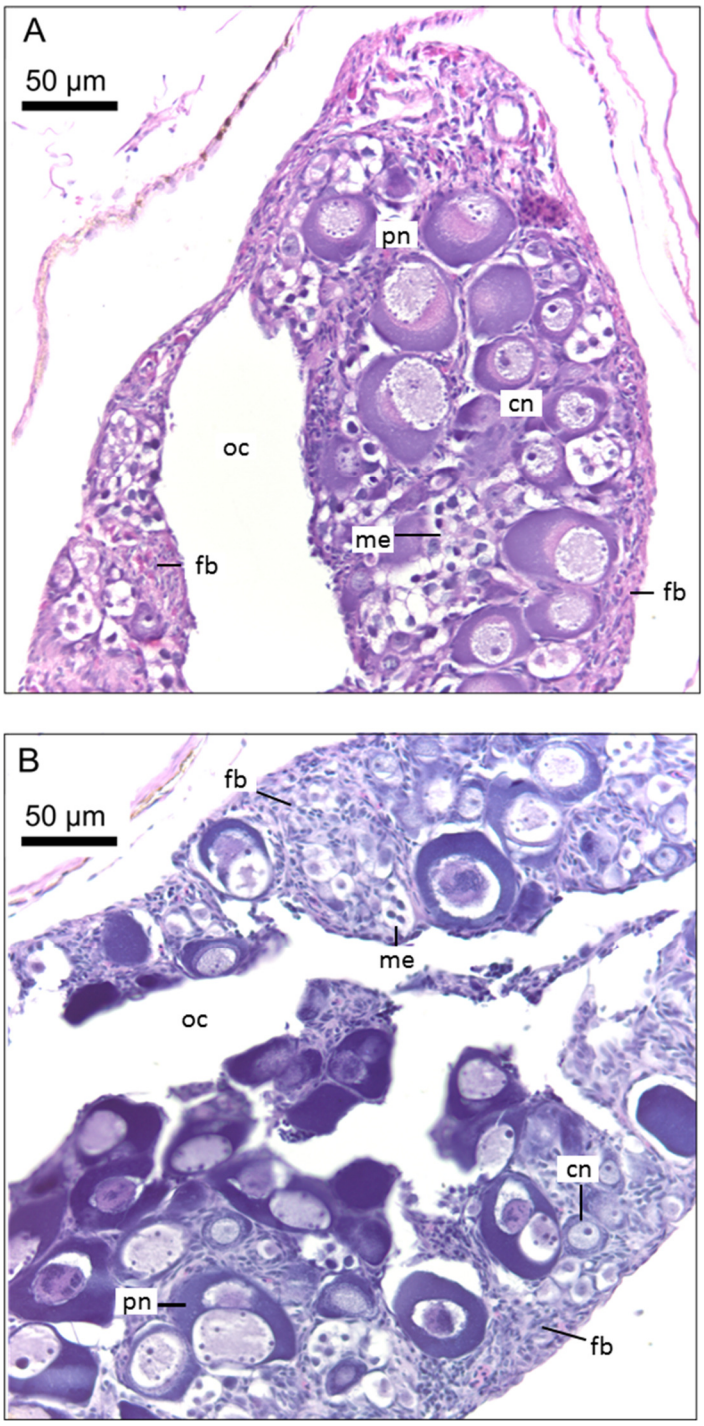


Abbreviations: **tb**, spermatic tubule – presumptive in A, and definitive in B; **gc**, germ cell

Among fish exposed to 3.3 ng/L EE2 (nominal; 4.3 ng/L mean measurement), ovarian phenotypes were observed in 100% of fish collected on test days 90 (n = 20) and 92% of fish collected on test day 120 (n = 12). A single fish collected on test day 120 had undifferentiated gonads. 38% of ovaries in these groups had an abnormal phenotype (Fig. 18A and B) characterized by smaller cross-sectional area (244-580 µm and 480-710 µm on days 90 and 120, respectively), fewer oocytes, and increased fibrosis around the perimeter, compared with control ovaries. Abnormal ovaries observed on test day 90 also had noticeably more clusters of early stage meiotic oocytes, and therefore appeared to be slightly delayed in development compared with control ovaries. The remaining ovaries in these groups were histologically indistinguishable from control ovaries.

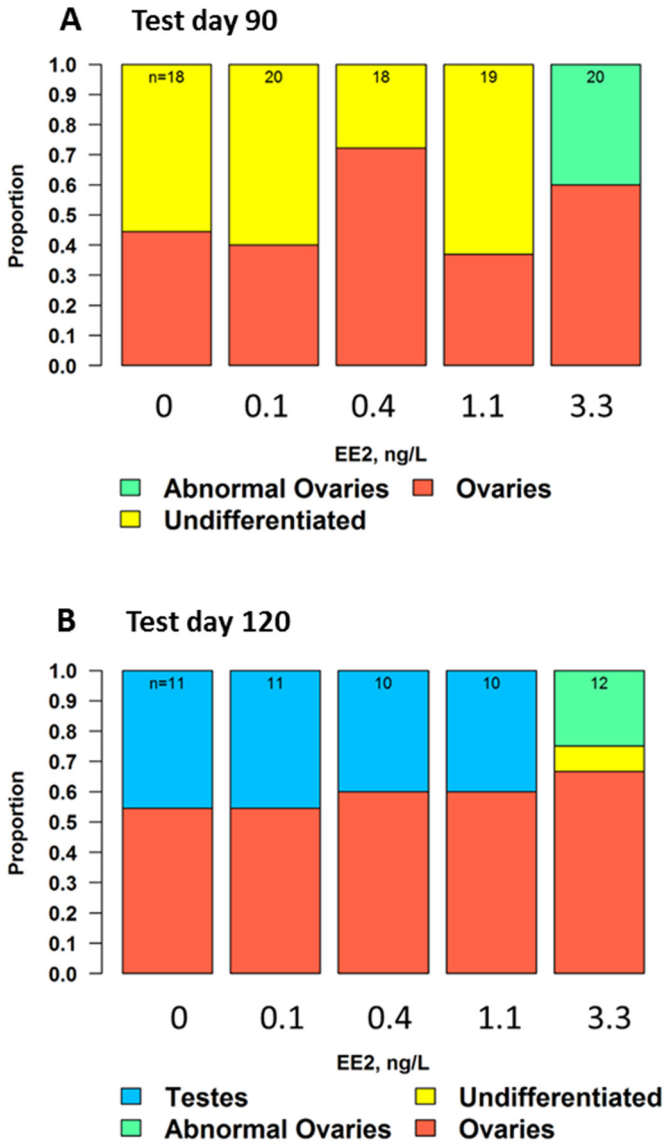
Among fish exposed to low concentrations of EE2 (0.1 and 0.4 ng/L nominal, and 1.6 ng/L measured), normal ovaries were present in 40%, 72%, and 37% of fish collected on test day 90, respectively (49% overall), and in 55%, 60%, and 60% of fish collected on test day 120, respectively (58% overall; Fig. 19A and B). No abnormal ovaries were observed in fish exposed to low concentrations of EE2. All fish from the low-EE2 concentration groups collected on day 90 that did not have ovaries, had undifferentiated gonads that were histologically similar to those in the control groups on that day (51% overall). Testicular differentiation was underway in 42% of fish collected from the low-EE2 concentration groups on day 120, and was histologically similar to the testicular tissues observed in the control groups on that day. No testicular oocytes, or any anomalies other than abnormal ovaries, were detected in the gonads of EE2-exposed fish.

Figure 18. Abnormal ovaries observed in fish exposed to 4.3 ng/L EE2 (mean measured concentration) for (A) 90 and (B) 120 days.



Abbreviations: **oc**, ovarian cavity; **me**, cluster of early stage meiotic oocytes; **fb**, fibrosis; **cn**, chromatin nucleolar oocyte; **pn**, perinuclolar oocyte

Figure 19. Proportions of gonadal phenotypes observed in control and EE2-exposed smallmouth bass collected on test days 90 (A) and 120 days (B). Fish were exposed to EE2 for 100 days, followed by 20 days in clean water. Nominal concentrations shown.



Ovaries and testes were identified by the presence of female- and male-specific germ cells and/or somatic structure. Undifferentiated gonads had early signs of testicular differentiation. Abnormal ovaries were characterized by smaller cross sectional area, fewer oocytes, and more fibrotic tissue compared with control ovaries.

Conclusions

The results of this study suggest that early life exposure to 4.3 ± 0.8 ng/L EE2 (mean ± SD of measured concentrations) caused presumptive sex reversal of male bass observed on test days 90 (n = 20) and 120 (n = 12), and that groups exposed to 1.6 ± 0.2 ng/L EE2 (mean ± SD of measured concentrations) developed gonads that were histologically indistinguishable from control groups. These results contrast slightly with previous work (Chapter III), in which fish exposed to measured concentrations ranging from 0.5 to 2.7 ng/L EE2 were presumably sex reversed. It is possible that the source of this discrepancy was related to differences in analytical methods that resulted in average exposure concentrations that were lower than nominal in Chapter III (experiment 1) and higher than nominal in Chapter IV (experiment 2), rather than an actual difference in fish sensitivity, but this cannot be determined from the available data. The present study also indicated that fish exposed to nominal concentrations of 0.1 and 0.4 ng/L EE2 developed normally, and that partial feminization in the form of TOs or mixed gonadal tissue was not caused by sub-sex reversal concentrations of EE2. It is possible that an intermediate effect concentration existed between this study's measured LOEC (4.3 ng/L EE2) and NOEC (1.6 ng/L EE2), although such a narrow effect threshold seems unlikely. However, a recent review has noted that, among all species tested, the threshold between EE2 exposures that caused minor feminization or decreased reproductive output and exposures that caused complete sex reversal was consistently steep: documented NOECs ranged from 0.3 to 1 ng/L based on reproductive output, and complete sex reversal occurred at 3 ng/L and above [73]. TOs have also been induced by some level of EE2

exposure in nearly every other species tested to date, including fathead minnow [8], medaka [12, 74, 75], roach [14], rare Chinese minnow [7], and rainbow trout [9]. Intersex has also been induced in carp (*Carpinus cyprio*) by exposure to estrogenic wastewater treatment plant effluent [76]. In wild populations, there are many examples of fish species that do not seem to develop TOs, even when coexisting in the same waters with species that have TOs [22, 23]. The work presented in Chapter II, as well as previous authors, have noted that wild collected smallmouth and largemouth bass do not exhibit the wide range of intersex observed in, for example, roach and darters, which have been observed with degrees of gonadal feminization ranging from mild (i.e. a few TOs) to severe (i.e. mixed gonadal sex) [24, 28, 31]. Mixed gonadal tissues or severely feminized testes have not been reported in any wild bass to our knowledge. This may suggest fundamental biological differences in bass, compared with other species, in terms of sensitivity and severity of responses to estrogenic exposure, and may be related to the lack of intermediate effects of EE2 observed in the present study.

V. Discussion

The studies described in Chapters II-IV have documented the occurrence of testicular oocytes (TOs) in populations of smallmouth bass living in more developed and less developed watersheds, characterized the pattern and stages of gonadal differentiation in laboratory-reared bass, and evaluated the effects of early life exposure to 17α-ethinylestradiol on bass gonadal development. These data and their interpretation increase our understanding of the conditions under which normal gonadal development, abnormal gonadal development, and TOs may occur, and provide a basis for evaluating the relationship between exposure to estrogenic compounds and development of TOs in this species.

Since it has become apparent that estrogenic compounds can cause sexual disruption in wild species, research has centered on detecting potential risks to fish populations, often by evaluating putative biomarkers of endocrine disruption. These efforts include recent studies showing estrogenic wastewater treatment plant (WWTP) effluent discharges have likely caused gonadal feminization in roach populations in rivers throughout the United Kingdom and darters in the Grand River in Ontario, Canada [24, 28, 31]. However, the occurrence of TOs in bass populations in the United States has been more difficult to interpret, and the evidence is not suggestive of a strong correlation between TOs and estrogenic exposure. Moreover, most studies have not comprehensively compared TO occurrence in populations that are likely to be exposed to estrogenic compounds with those from relatively pristine sites.

This study has made use of the many minimally developed lakes in NE Minnesota to conduct a thorough evaluation of bass populations without evident exposure to estrogenic compounds. Based on two hypotheses, that human activities (especially WWTP effluent and agriculture) increase the likelihood of estrogenic exposure, and that estrogenic compounds increase TO prevalence and/or density, it was predicted that fish from more developed watersheds would have more TOs than fish from less developed watersheds. While this was confirmed to a degree, our findings discussed in Chapter II suggested that TO prevalence and density were not strongly related to WWTP presence or human population density, and notably, that factors unrelated to estrogenic exposure were also correlated with the presence of TOs. Two possible interpretations of these results are 1), TOs were caused by estrogenic exposure from unknown sources or by unknown agents not predicted by the presence of human development, or 2), bass TOs may develop spontaneously.

Many researchers have noted that the prevalence of spontaneous TOs is unknown in smallmouth bass, as in most other fish species. Therefore, the main goals of the laboratory portion of this project were to produce histological descriptions of early gonadal development in smallmouth bass reared under controlled conditions, to identify the developmental stages at which abnormalities can be detected, and to evaluate the likelihood that TOs can develop as a part of normal gonadal differentiation or occur spontaneously during early life stages. Our findings indicate that intersex and testicular oocytes could have been detected, at the earliest, on test day 92 (presumably less than 99 days post hatch) in experiment 1 (Chapter III) or test day 120 (presumably less than 127

days post hatch) in experiment 2 (Chapter IV), based on the presence of clearly defined male-specific characteristics and the co-occurrence of late-stage primary oocytes in females in the same age groups. TOs were not observed in any of the control males in these, or later, age groups (n = 50), suggesting that spontaneous TO development may be rare (< 2%). However, TO prevalence was 8% (n = 24) in adult male smallmouth bass collected from the same minimally developed lake from which yolk-sac fry were collected for these studies. The likelihood that TOs were not present in any of the laboratory-reared males, if 8% is the natural background rate, is low; however, the fish used for those experiments were collected from only 6 nests, so it is possible that the laboratory-reared fish were offspring of parents with low disposition to TOs. In laboratory cultures of non-hermaphroditic species (e.g. medaka (*Oryzias latipes*), fathead minnow, roach (*Rutilus rutilus*), and carp (*Cyprinus carpio*), the spontaneous occurrence of intersex has been reported to range from >1% to 21% [24-27]. It also should be noted that the presence of spontaneously developing TOs, and the existence of individuals with varying levels of disposition to spontaneous TOs, is hypothetical, since we do not understand the mechanisms by which TOs arise.

The other key goals of the laboratory studies were to evaluate the effects of early life EE2 exposure on gonadal development, and, if possible, to relate those findings to observations of TOs in wild adult fish. The results reported in Chapters III and IV indicated that all fish exposed to moderately high EE2 concentrations (mean measurements of 1.3 and 4.3 ng/L, respectively) developed ovaries, while the gonads of fish exposed to lower, more environmentally realistic concentrations (nominally 0.1 and

0.4 ng/L) developed into histologically normal ovaries or testes in approximately a 1:1 ratio. TOs were not observed in any fish exposed to EE2, although a single fish exposed to EE2 in experiment 1 (Chapter III) was intersex, with clearly defined regions of both testicular and ovarian tissue within the gonads. The concentrations at which severe feminization occurred (presumed male-to-female sex reversal) have seldom been detected in environmental samples, and are predicted to occur rarely in natural waters, even under worst-case scenarios [16]. This suggests it is unlikely that EE2 will cause wide-spread sex reversal in wild smallmouth bass populations.

The results of these studies, especially the lack of TOs observed in laboratory fish exposed to very low concentrations of EE2, do not allow for an unambiguous interpretation of the presence of TOs in adult wild fish. It is generally accepted that adult fish are less sensitive to endocrine disrupting compounds than juvenile fish; therefore, it might be hypothesized that concentrations of EE2 that cause complete sex reversal in juveniles may cause only partial feminization, or TOs, in adult fish. If this is occurring in wild populations, female-skewed sex ratios would also be expected in lakes with the highest TO prevalences. To test this theory, juvenile phenotypic sex ratio should be evaluated in populations with high TO prevalence. In a preliminary study concurrent with this research, young-of-year smallmouth bass were collected from a more developed watershed with high adult TO prevalence (Lake Shagawa; n = 11); and a less developed watershed with low adult TO prevalence (Pike Lake; n = 24). Juvenile gonad phenotypes were evaluated according to previously described methods. Fish weights and lengths varied from 1.4-17.4 g and 45-117 mm, respectively, corresponding roughly to the

weights and lengths of fish that were reared in the laboratory for 92-180 days. Histological evaluation showed that samples collected from Lake Shagawa included young-of-year bass with ovaries, undifferentiated gonads, and testes, but no abnormal phenotypes or TOs were observed (Appendix C). The young-of-year bass collected from Pike Lake had either ovaries or undifferentiated gonads. These results suggest that fish from both of these populations were undergoing normal gonadal development, and that the young-of-year sex ratio in the population with high adult TO prevalence was not strongly female-biased, although sample numbers were too low for robust assessment of sex ratio.

Because these experiments did not extend beyond 180 days, we have no empirical evidence related to the long-term outcomes of early life sex reversal, or of early life exposure to sub-sex reversal EE2 concentrations. One possibility is that bass may be able to recover from sex reversal by resuming testicular development and spermatogenesis, and suppressing ovarian characteristics and, perhaps incompletely, oogenesis. Under this hypothesis, some TOs and/or oogonia which later develop into TOs may remain within otherwise normal testicular tissue. However, the fact that the abnormal ovary phenotype persisted even after cessation of EE2 exposure, as reported in Chapter III, suggests that sex reversal may have been permanent, as it is in most other fish species [6, 12]. One known exception is zebrafish males that were sex reversed by a short exposure to EE2 during early life stages, and were shown to either partially resume testicular development, exhibited by intersex gonads, or to fully recover and develop normal, functional testes, depending on whether they were exposed to a high or low EE2 concentration [77]. It has

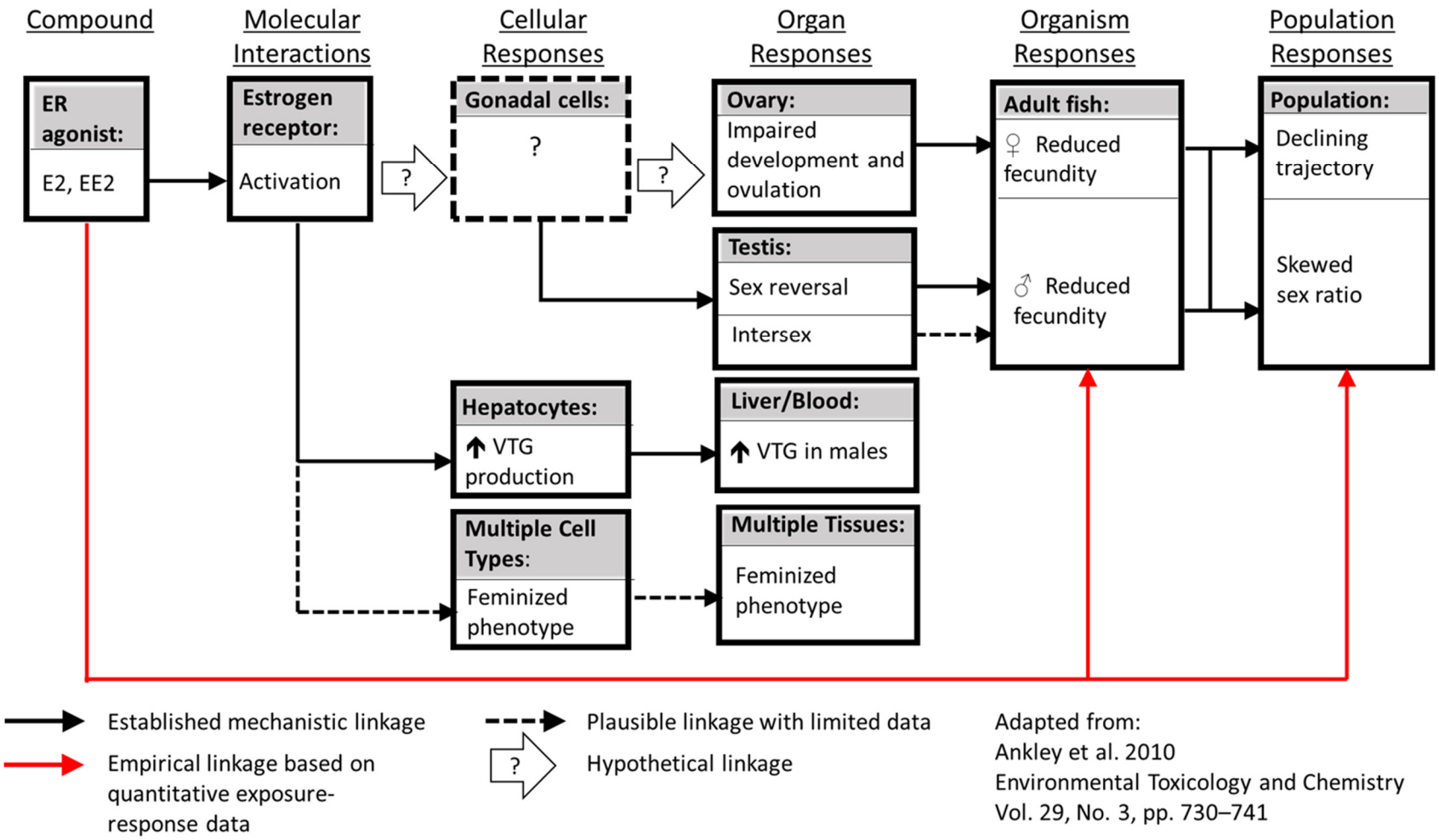
been suggested that the potential for male zebrafish to recover from early life sex reversal is related to their undifferentiated gonochorism type of gonadal ontogeny, because testicular development, even under normal conditions, involves the reorganization of existing ovarian tissues [44]. Conversely, it may be argued that smallmouth bass and other differentiated gonochorists, in which normal development does not include an ovary-to-testis transition period, may be less likely to recover from sex reversal.

Another possible explanation for the discrepancy between TOs in adult wild fish and the absence of TOs in lab-reared juveniles is that TOs may be a delayed effect of estrogen exposure during early life stages, and do not appear until adulthood. This could occur if EE2-exposed male fish developed testes that were histologically normal except for some proportion of germ cells that differentiated into oogonia instead of spermatogonia, which would have been undetectable by the methods of the present studies. Testing this would require the development of an assay for identifying oogonia in histological sections, such as the *in situ* hybridization assay for ovary structure protein 1, which is being developed as a biomarker for intersex in medaka [78]. Hypotheses related to the long term outcomes of sex reversal and exposure to very low EE2 concentrations could also be tested more directly with longer test durations; however, there are significant challenges associated with rearing smallmouth bass for extended periods.

Further understanding of the signaling pathways involved in gonadal differentiation and germ cell development may improve our ability to interpret the presence of TOs in wild fish populations. Gonadal differentiation and the development

of germ cells are influenced by steroid hormones, sex steroids, steroidogenic enzymes, and hormone receptors, which are directed by a set of genes that fairly well-conserved among non-mammalian vertebrates [79]. It is thought that estrogen receptor-mediated alterations in signaling pathways can lead to sex reversal and intersex in male fish, but these pathways are complex. As shown on a current representation of the adverse outcome pathway for estrogen receptor activation, the responses of gonadal cells to estrogen receptor activation which lead to alterations in the testis are not completely understood (Fig. 20, [18, 80]). One potential key event in this pathway is increased expression of CYP19 (the gene that codes for the cytochrome P450 enzyme, aromatase, which catalyzes the conversion of testosterone into estradiol) [81], which has been induced in male fish by exposure exogenous estrogen, and has also lead to increased expression of estrogen receptors [82] and inhibition of testosterone expression in the testis [83]. Estrogenic exposure has also caused downregulation of testis differentiation genes *amh* and *dmrt1* in fathead minnows [80], which are normally inhibited only in genetic females, suggesting that male-to-female sex reversal may occur when male-specific pathways are inhibited, allowing female-specific pathways to shift the balance of endogenous steroidogenesis from androgens to estrogens. In Chapters III and IV we reported that abnormal characteristics were first observed in the ovaries of EE2-exposed fish at the same time point at which testicular differentiation was first observed in control group males, suggesting that male-specific signals may not have been completely repressed during the development of ovaries in sex-reversed males.

Figure 20. Adverse outcome pathway for estrogen receptor activation.



Recent studies have implied that the same set of pathways may be involved in the partial feminization of testes, including the development of ovarian ducts or testicular oocytes [78, 84]. These studies suggest that TOs may occur when germ cells differentiate into oogonia instead of spermatogonia. A small population of germ cells that are maintained in the testes are responsible for replenishing the population of spermatogonia for each reproductive cycle. In seasonal spawners, the reproductive cycle includes a period of gonadal regression immediately after spawning, followed by recrudescence, in which the germinal epithelium is rebuilt in preparation for the next cycle of gametogenesis. It has been suggested that this process may involve a reactivation or intensification of the same pathways responsible for initial gonadal differentiation, and is therefore a potential window of sensitivity during which bipotential germ cells in males are susceptible to exogenous estrogen signals that may induce differentiation into oogonia instead of spermatogonia [84]. Consistent with this idea, results reported in Chapter II indicate that TO prevalence and density were significantly higher in the fall, when fish were undergoing recrudescence.

The reproductive success of smallmouth bass exposed to EE2 concentrations below those that can cause sex reversal, and of wild smallmouth bass with TOs, is unknown. Very few studies have measured the reproductive success of partially feminized male fish, but available data suggest it can either be similar to control males (e.g. medaka; [12]), or slightly decreased (e.g. fathead minnow [26, 85], roach [86]). One study also found slightly decreased sperm motility in bass with TOs, but reproductive success was not tested [39]. Another study showed that reproductive potential was

decreased in estrogen-exposed threespine stickleback (*Gasterosteus aculeatus*) males that had histologically normal gonads [87].

This study has also shown that smallmouth bass can be used for laboratory studies, although substantial challenges exist, and that gonadal differentiation (though not maturity) in both sexes may occur within 120 days. Despite the logistical difficulties associated with rearing non-standard species in a laboratory, such studies can contribute to assessments of environmental risks to wild populations.

Overall, these studies do not suggest that TOs in wild adult smallmouth bass are related to early life exposure to EE2, although the long-term outcomes of EE2 exposure are unknown. Considering the present state of understanding that indicates only weak correlations between wild bass TO occurrence and human activities, we conclude that estrogenic compounds are likely not the unique cause of TOs, and that the presence of TOs alone is not a suitable biomarker of estrogenic exposure in wild smallmouth bass. However, the high degree of sensitivity to EE2 in laboratory exposures, along with the frequent occurrence of TOs in wild fish, suggest future opportunities to use smallmouth bass as a model for elucidating relationships between estrogen receptor agonism, TOs and gonadal feminization, and adverse outcomes at the individual and population levels. There will also likely be ongoing public interest in documenting the occurrence of TOs in wild bass populations, given the continuing awareness of risks of EDCs, the high cost of wastewater treatment facility upgrades, and the cultural significance of these species. The methods and evaluation criteria outlined by the studies presented here may serve to facilitate those interests.

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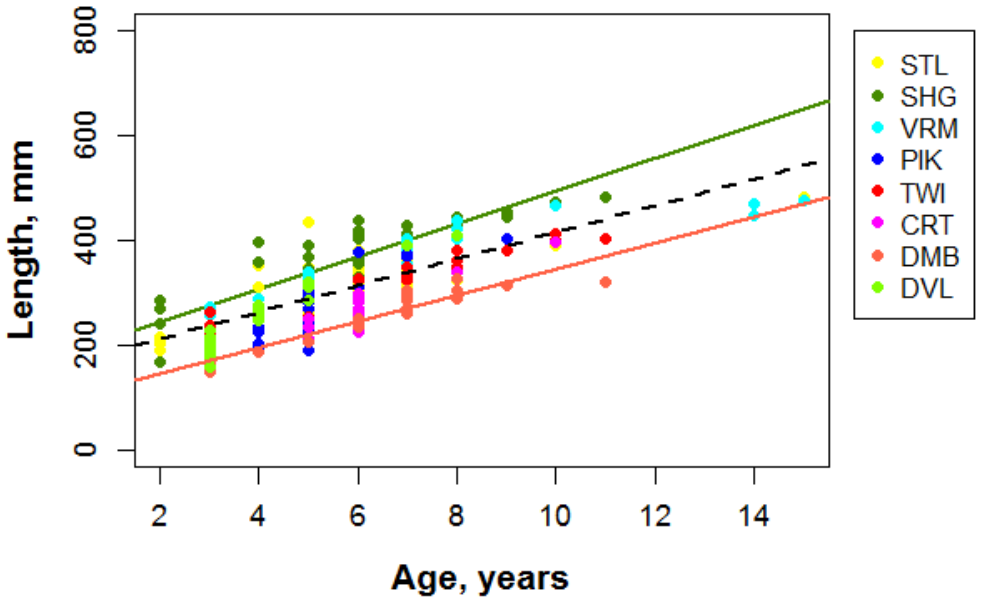
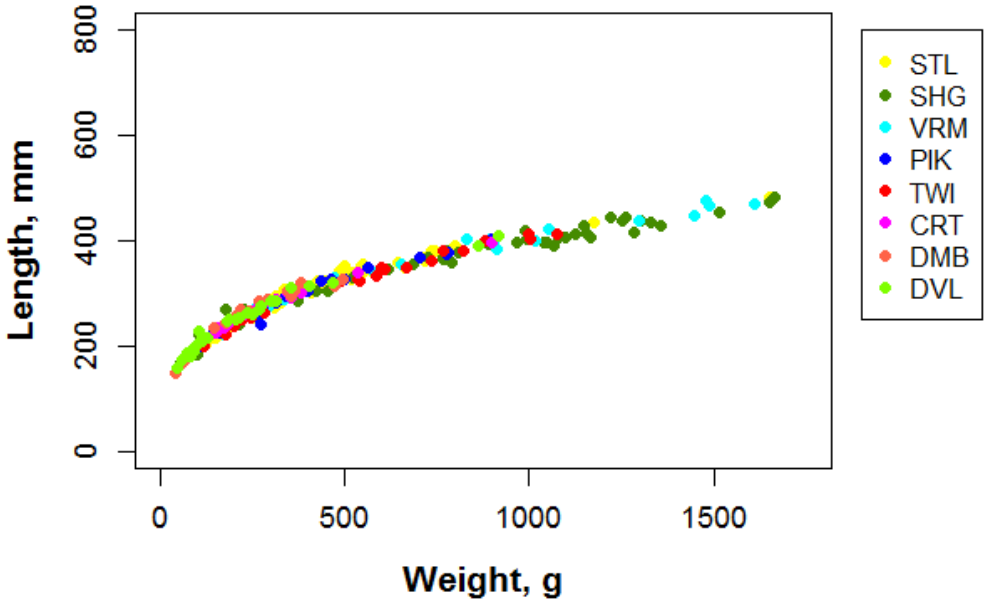
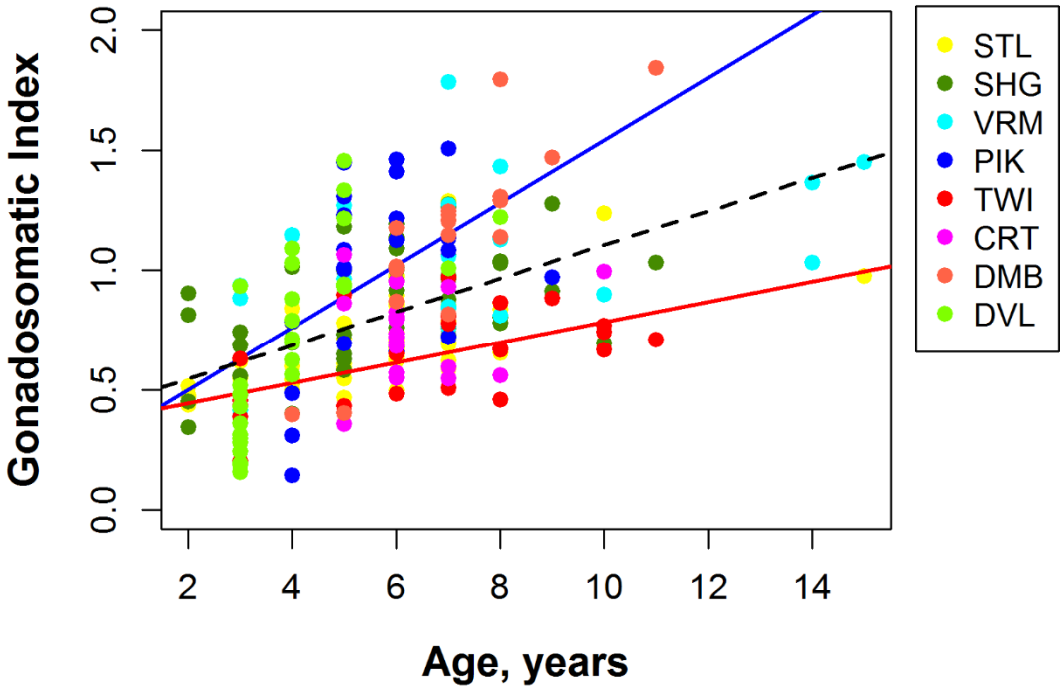
APPENDICES

Appendix A. Scatterplots of selected pairs of biometrics in smallmouth bass collected from eight study sites. Dashed lines indicate the overall trend line for all data points. Colored lines indicate trend lines for selected individual sites (see text for site IDs). In general, only minor differences were noted among the 8 study sites.






Gonadosomatic Index
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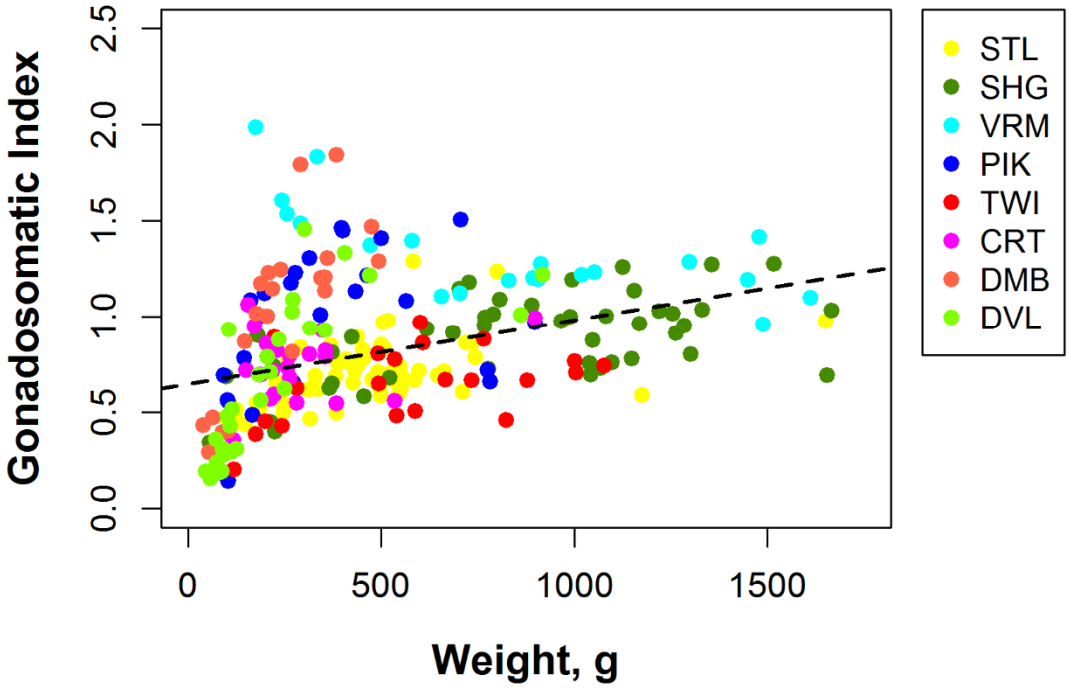
0.5
1.0
1.5
2.0
2
4
6
8
10
12
14
Age, years
STL
SHG
VRM
PIK
TWI
CRT
DMB
DVL


Gonadosomatic Index
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0
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1000
1500
Weight, g
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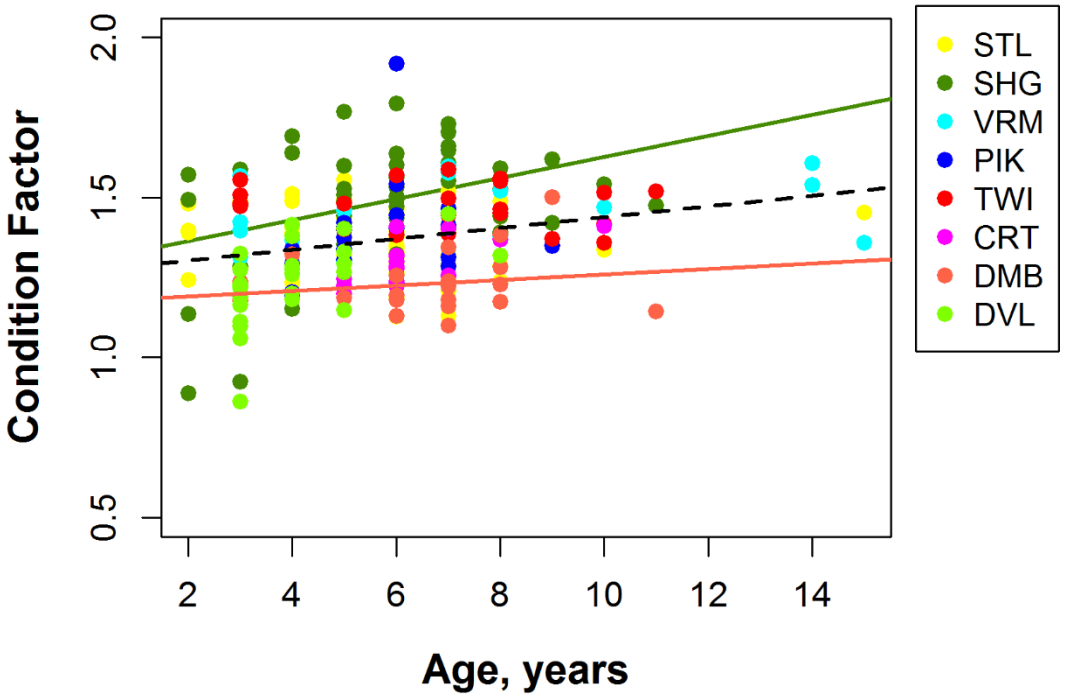
SHG
VRM
PIK
TWI
CRT
DMB
DVL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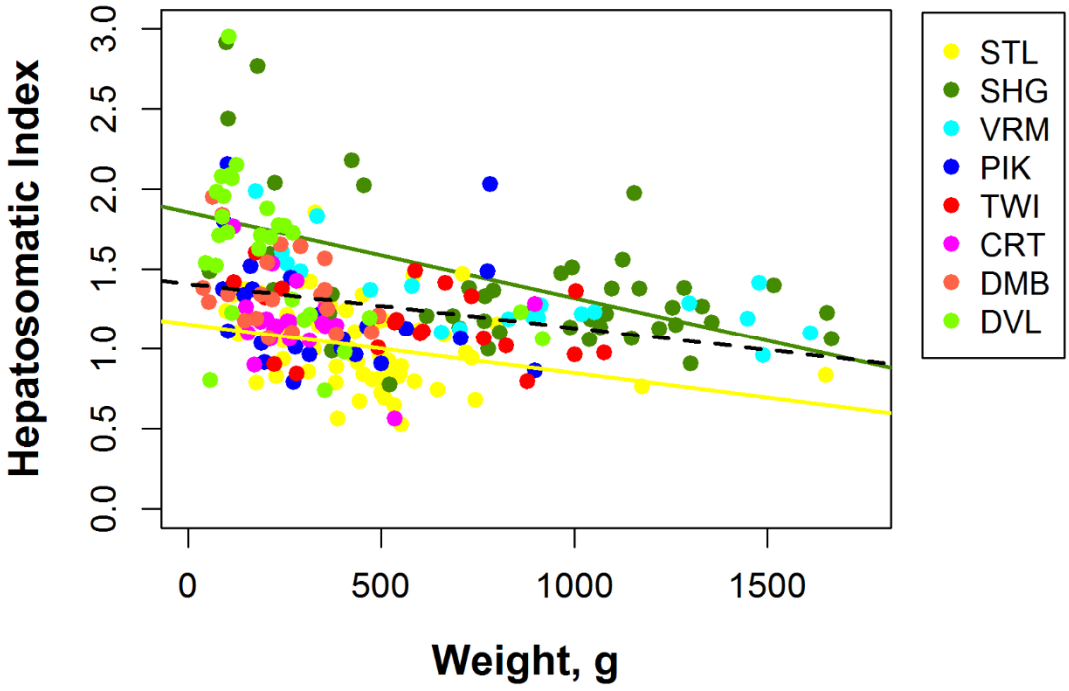
1.0
1.5
2.0
2
4
6
8
10
12
14
Age, years
STL
SHG
VRM
PIK
TWI
CRT
DMB
DVL


Hepatosomatic Index
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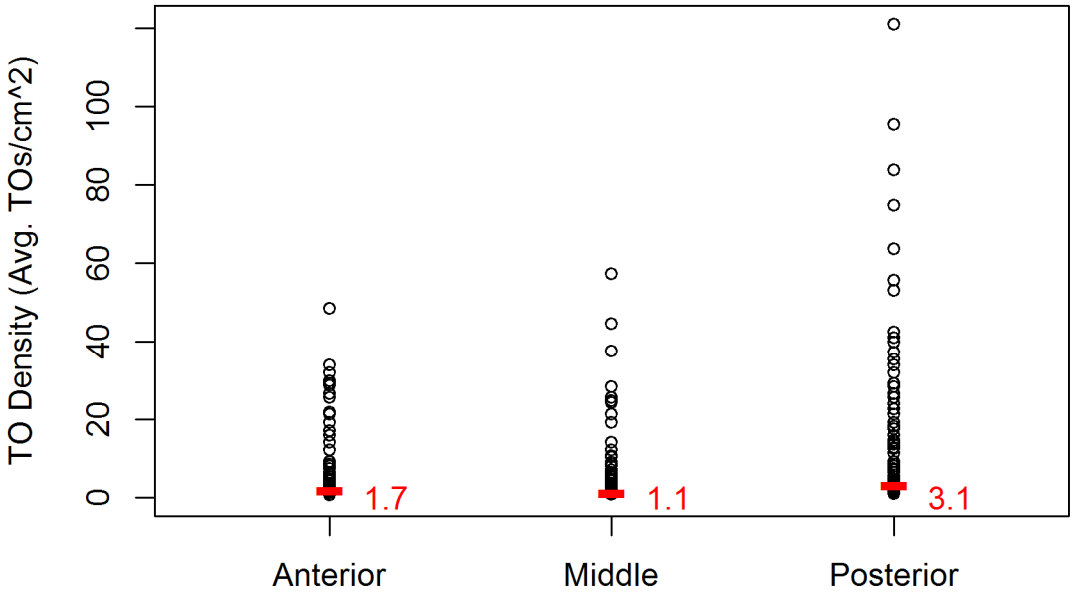
2.0
2.5
3.0
0
500
1000
1500
Weight, g
STL
SHG
VRM
PIK
TWI
CRT
DMB
DVL

Appendix B. TO densities observed in the anterior, middle, and posterior regions of smallmouth bass testes. Means shown in red.



Appendix C. Number of each gonadal phenotype present among smallmouth bass young-of-year. Weights and lengths are reported as mean (std. dev.)

Study Site	Weight, g	Length, mm	Ovaries	Undifferentiated gonads	Testes	*n*
Lake Shagawa	6.6 (5.9)	71 (24)	6	18	0	24
Pike Lake	2.3 (0.6)	54 (5)	3	4	4	11